Spiny lobster Lifecycle

Berried females

Eggs

Nauplisoma

Instar I phyllosoma

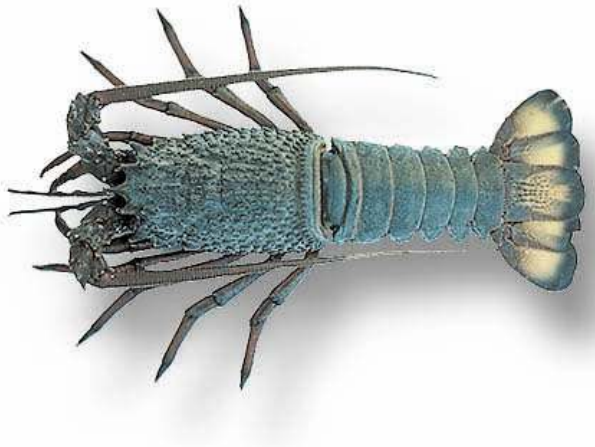
Final instar phyllosoma

Puerulus

Juvenile

Adult

PHYSIOLOGICAL RESPONSES TO DIFFERENT ENVIRONMENTAL AND CULTURE CONDITIONS DURING ONTOGENY OF THE SPINY LOBSTER, *Sagmariasus verreauxi*



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Abstract

Very little is known about the metabolic and biochemical physiology of spiny lobsters as they develop. An improved understanding of the physiological responses of spiny lobsters to different environmental and culture conditions during ontogeny is essential for gaining a better understanding of environmental influences on wild populations and for their successful propagation. This study addresses important gaps in our knowledge by examining stage-specific changes in metabolic rates, ammonia-N excretion rates, thermal tolerance thresholds, and whole body and haemolymph biochemistry of larval and juvenile *Sagmariasus (Jasus) verreauxi* in order to observe these physiological parameters through ontogeny. Automated intermittent flow-through respirometry was used to measure the aerobic metabolism of larval and juvenile lobsters accurately. Whole body biochemical analysis was used to examine energy storage and utilisation of phyllosoma. Haemolymph biochemistry was used to determine the thermal tolerance thresholds of juvenile lobsters.

The effect of temperature change on the final instar (instar 17) was examined to assess whether it could serve as a cue for metamorphosis and it was found that temperature affected routine metabolic rate (R_r), but did not alter instar duration, and therefore is not a cue for metamorphosis. Fewer phyllosoma, however, completed metamorphosis and progressed to the puerulus stage at sub-optimal warm temperatures compared to cooler temperature. The effect of culture density was examined from hatch to pueruli. High culture density reduced growth (dry mass) and development (moult increment) of phyllosoma. There was a shift in metabolism via energy storage and utilisation of instar 17 phyllosoma in preparation for the morphological changes associated with metamorphosis. There was also an

accumulation of lipid reserves during larval development that fuelled metamorphosis and the non-feeding puerulus stage.

The aerobic scope of juvenile lobsters was determined through chasing lobsters by hand until the lobster was exhausted and did not respond to further stimuli along with the effects of handling, anaesthesia, and activity on the oxygen consumption rate ($\dot{M}O_2$) and ammonia-N excretion rate. Handling caused a relatively minor increase in $\dot{M}O_2$ and anaesthetics reduced activity of lobsters, but did not reduce $\dot{M}O_2$ or recovery periods from force feeding or handling. *S. verreauxi* juveniles have a narrow aerobic scope. Increased $\dot{M}O_2$ from anaesthesia and activity uses a large proportion of energy within the metabolic scope that could otherwise be utilised for other physiological functions. Thermal tolerance thresholds were examined in different sized juvenile lobsters. Sudden changes in haemolymph O_2 concentrations with water temperature indicated large lobsters have a higher optimum water temperature than small lobsters. Maximum attainable rates of standard metabolism indicated the upper critical temperature (T_c) for juvenile *S. verreauxi*, characterised by the onset of anaerobic metabolism. Juveniles utilised lipid as an energy substrate at optimal temperatures, but shifted towards protein catabolism at temperatures above their thermal tolerance range.

This research revealed the long larval phase of *S. verreauxi* is essential for accumulating lipid reserves to fuel later larval stages and provided a more complete picture of the environmental and culture requirements of spiny lobsters during ontogeny, particularly for the rarely studied late phyllosoma stages. It also established that induced stress uses a large proportion of the aerobic scope in juvenile lobsters, which limits their ability to perform aerobically and deal with additional physiological challenges. The thermal tolerance of *S. verreauxi* juveniles identified

the potential to expand their geographic distribution, which may have a large impact on benthic community structures and dynamics through competition for resources with existing lobster populations and other animals within the ecosystem. This may also impact local economies, particularly valuable local southern rock lobster fisheries.

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GENERAL INTRODUCTION

Chapter I

1.1 Introduction

To my knowledge the metabolic and biochemical physiology of spiny lobsters has never been studied through the entire course of larval and juvenile development. High mortality during larval development and early juvenile stages highlights the requirement for further research into defining optimal environmental and culture parameters as a first step in the successful propagation of spiny lobsters. This thesis describes research into the physiological responses to different environmental and culture conditions of *Sagmariasus (Jasus) verreauxi* through ontogeny to extend our knowledge of the tolerance of spiny lobsters to culture conditions and elucidate key areas within the lobster lifecycle. Numerous exogenous and endogenous factors affect the physiological processes of spiny lobsters at different stages of development. Therefore, the overall aims of this study were:

- To analyse the physiological response to a change in water temperature during the final instar and to determine if a change in temperature is a cue for metamorphosis in *S. verreauxi* (Chapter 2).
- To examine the effect of culture density on the growth, development, routine metabolic rate (R_r), ammonia-N excretion rate, and energy storage and utilisation of *S. verreauxi* from hatch to puerulus in order to evaluate the energetic demands through larval development (Chapter 3).
- To examine the effect of culture density on the growth and biochemical composition of *S. verreauxi* through the entire larval phase to better understand the energy storage requirements of late stage phyllosoma prior to metamorphosis (Chapter 4).

- To determine aerobic scope and assess the effects of handling, anaesthesia, and activity on the oxygen consumption rate ($\dot{M}O_2$) and ammonia-N excretion rate of juvenile *S. verreauxi* in order to evaluate the proportion of the aerobic scope each of these factors takes up (Chapter 5).
- To define the thermal optimum and critical temperature thresholds of two size classes of *S. verreauxi* juveniles in order to identify potential climate effects on their distribution (Chapter 6).

S. verreauxi is an excellent species for this research; they are relatively fast-growing and robust compared to other spiny lobster species, they have a wide thermal tolerance and high commercial value. The aim of this chapter is to provide background information on exogenous and endogenous factors affecting the metabolic and biochemical physiology of spiny lobsters.

1.2 Lifecycle

The spiny lobster *S. verreauxi* (Decapoda: Palinuridae) is a warm temperate lobster species that is endemic to the north of New Zealand and south-east Australia (George, 1966; Booth, 1979; Booth and Ovenden, 2000). *S. verreauxi* is known colloquially as the green or packhorse lobster in New Zealand and the eastern rock lobster in Australia (Kittaka et al., 1997) and supports important regional fisheries in both these countries (Montgomery and Craig, 2005). The lifecycle of *S. verreauxi*, as for all spiny lobster species, includes an extended zoeal stage classified as phyllosoma.

Phyllosoma are transparent, flattened and leaf-like in shape with 3-5 pairs of biramous appendages (Ikeda et al., 2011). The phyllosoma life-stage for *S. verreauxi* can last up to 12 months in the wild (Booth and Phillips, 1994) or 8 months in the laboratory, with 11 distinct morphological stages and 17 moults, which are classified

as instars (Kittaka et al., 1997). Phyllosoma are raptorial feeders, feeding on zooplankton (Phleger et al., 2001). However, the diet of phyllosoma changes as they progress through the larval phase, with early stage phyllosoma feeding mainly on zooplankton high in eicosapentaenoic acid (EPA) such as diatoms, and late-stage phyllosoma feed on plankton rich in docosahexaenoic acid (DHA) such as dinoflagellates, cnidarian jellies, and krill (Nichols et al., 2001; Phleger et al., 2001; Jeffs et al., 2004). Phyllosoma have limited swimming ability and are transported by offshore currents, before the final instar (instar 17) phyllosoma metamorphose into a completely transparent puerulus beyond the edge of the continental shelf (Booth, 1986).

The puerulus is a non-feeding stage and relies entirely on stored energy reserves to swim across the continental shelf towards the coast (Jeffs et al., 1999) before finally settling in shallow water and moulting to the benthic juvenile lobster within about three weeks (Menzies and Kerrigan, 1979; Phillips; 1981; Jeffs et al., 2001a). *S. verreauxi* can only recruit to the adult population following successful transition from the pelagic to the benthic environment.

Juvenile spiny lobsters inhabit inshore waters generally associated with reefs. *S. verreauxi* juveniles reach maturity at >120 mm carapace length (CL) (Booth, 1997) which takes around 5 years from hatch, although males may mature earlier than females. Small juveniles feed mainly during the night on ophiuroids, isopods, and bivalves. Larger juveniles and adult spiny lobsters feed primarily on bivalves, crabs and other smaller crustaceans, sea urchins and gastropods (Edmunds, 1995).

Adult females carry broods of eggs during the breeding season, which is a few months long in *S. verreauxi* (Booth and Phillips, 1994). Breeding in *S. verreauxi* occurs once each year and females have a high fecundity (>200,000 eggs per female),

releasing eggs in batches over a period of 2-3 days. Females migrate offshore (about 20 km) in spring (October) after they have become egg bearing in shallow (<60 m) inshore waters where hatching occurs during summer (December and January) (Booth, 1986). Adults move inshore again in winter or early spring to moult and mate (Booth, 1997). The maximum CL observed in the wild is around 250 mm for both males and females (Montgomery et al., 2009).

1.3 Moult cycle

The stages within the moult cycle have been described in several lobster species and contain four major moult stages, defined as postmoult (*A*-*B*), intermoult (*C*), premoult (D^0-D^4), and ecdysis (*E*) (Turnbull, 1989; Musgrove, 2000). During postmoult, crustaceans take up a large amount of water and, consequently, increase rapidly in wet mass and expand their cuticle. Once the cuticle has expanded and the final size is attained, the cuticle is strengthened by calcium deposition. The postmoult usually accounts for approximately 2-3% of the total moult cycle duration (Musgrove et al., 2000). Intermoult is a period of maximum growth and minimal development where the endocuticle is fully developed and reaches maximum thickness. The length of the intermoult phase increases with successive moults and accounts for around 80-90% of the total moult cycle duration (Musgrove et al., 2000). When comparing metabolic rates and biochemical composition of lobsters, it is important to select individuals in the intermoult period, where development is minimal and metabolic rates are stable. Premoult is characterised by the epidermis starting to withdraw from the cuticle, the invagination of epidermal tissues, separation of setae from the old cuticle, formation of new structures, significant increase of the surface area in pre-existing body parts, and appearance of a new cuticle (Anger, 2001). In percentage terms, premoult ranges from 7-15% of the moult cycle duration (Musgrove et al.,

2000). Ecdysis refers to the process of moulting in crustaceans where the old cuticle ruptures between the carapace and the pleon and the animal withdraws itself from the old exoskeleton in a process that takes less than a minute (Anger, 2001). Ecdysis has long been recognised as a critical point in the development of crustacean larvae; it requires a large amount of energy due to the many morphological and physiological changes that occur during premoult; and can result in death in weak larvae unsuccessfully attempting to moult (Anger, 2001).

1.4 Density

Density is an important factor in larval rearing of crustaceans because it may contribute to excessive energy expenditure through increased physical interactions with prey and other phyllosoma. This increased interaction may lead to entanglement of phyllosoma or physical damage, which impairs their ability to actively capture prey (Mikami, 1995; Smith and Ritar, 2006). Intensive larval culture at high densities may also result in accumulation of toxins, such as ammonia, that are potentially detrimental to phyllosoma (Piamsak and Somkiate, 1980; Emmerson and Andrews, 1981; Millamena et al., 1991). Larval rearing of crustaceans has often occurred at high density without testing the effects of these densities on growth and survival or physiological parameters (Emmerson and Andrews, 1981). Increased competition for available food at high stocking densities may limit feeding success (Minagawa and Murano, 1993; Mikami, 1995). This reduced feed intake at high culture densities may also affect the viability of phyllosoma in later stages and their ability to survive and successfully complete metamorphosis (Smith and Ritar, 2006). Although the effects of stocking density are reported to vary between lobster species (Johnston et al., 2006) and life-stages (Booth and Kittaka, 2000), the effects of stocking density have not

been investigated previously during ontogeny of any lobster species and was therefore examined in Chapters 3 and 4.

1.5 Metabolism

Metabolic physiology of spiny lobsters has never been studied through the entire course of larval and juvenile development. This is mainly attributed to the difficulties of culturing phyllosoma through their long larval phase in order to produce juveniles (Ritar et al., 2003) and difficulties associated with capturing undamaged phyllosoma from the wild (Ikeda et al., 2011). Previous studies on metabolism have been restricted to early and mid-instar phyllosoma and small juvenile stages (Bermudes and Ritar, 2004; Bermudes et al., 2008; Ikeda et al., 2011). Information on the growth, $\dot{M}O_2$ and ammonia-N excretion during ontogeny is of particular interest because the ratio of oxygen respired to ammonia-N excreted represents the metabolic substrate for energy production and energy partitioning (Regnault, 1981). Evaluation of growth (dry mass; DM, and total length; TL) and metabolism ($\dot{M}O_2$ and ammonia-N excretion rate) may also be useful in providing information for understanding energy demand and nutritional condition during ontogeny of spiny lobsters (Ikeda et al., 2000; Anger, 2001) and was investigated in response to different stocking densities in Chapter 3.

1.5.1 Aerobic respiration

Spiny lobster phyllosoma initially rely on oxygen uptake by passive diffusion across their body surface until gill buds develop in later phyllosoma stages (Kittaka et al., 1997). Juveniles and adults rely on the uptake of oxygen via fully developed gills. Filamentous gills in juveniles and adults are contained within the gill chambers located below the carapace within the lower lateral section of the cephalothorax

(Kemp et al., 2009). The morphological and physiological properties of oxygen transport tissues and cells have been reviewed extensively by Mangum (1983), Mantel and Farmer (1983), McMahon and Wilkens (1983), Felgenhauer (1992), Taylor and Taylor (1992), and Charmantier (1998). Oxygen is circulated through the haemolymph via oxygen carriers called haemocyanin (Mangum, 1983).

Oxygen consumption (aerobic respiration) is one of the most commonly used measures for assessing energy metabolism in aquatic animals (Perera et al., 2005). The rate of oxygen consumption is strongly associated with the overall rate of aerobic metabolic processes (metabolic rate), and when converted to its energy equivalents is referred to as indirect calorimetry (Brett and Groves, 1979; Brafield, 1985). Metabolic rate is often measured at various levels of activity (Cockcroft and Wooldridge, 1985). These levels are standard (minimum level compatible with life), routine (spontaneous activity) and active (sustained maximum activity) (see Fig. 1.1). Standard metabolic rate (R_s) is measured in a post-absorptive, non-reproductive resting animal. Values of R_r can vary considerably due to unquantified activity and feeding. Even though R_r is not as useful as R_s for measuring particular physiological functions, R_r provides a good indication of the average oxygen requirements of an organism (Lankin et al., 2008). Active metabolic rate (R_{active}) is the highest rate of energy expenditure and is most accurately measured using chase protocols to exercise crustaceans to near exhaustion (Booth and McMahon, 1992; Jimenez et al., 2008). Following exercise, animals (including lobsters) typically display an increase in $\dot{M}O_2$ above R_s, which is referred to as excess post-exercise oxygen consumption (EPOC) (Lee et al., 2003). The maximum $\dot{M}O_2$ under this situation is equivalent to the aerobic capacity (Steffensen, 2005). There are many factors that have been shown to affect $\dot{M}O_2$ in lobsters including body mass, temperature, activity, feeding, handling,

dissolved oxygen level, salinity, diurnal rhythm, and moulting cycle (Buesa, 1979; Crear and Forteath, 2000; Diaz-Iglesias et al., 2004). However, $\dot{M}O_2$ has not previously been measured through ontogeny of any spiny lobster species and was therefore measured in response to temperature change (Chapter 2) and stocking density (Chapter 3) in the current study.

Previous measurements of $\dot{M}O_2$ in crustaceans have most frequently used static (closed) respirometry systems (Dall, 1986; Steffensen, 1989; Bermudes and Ritar, 2004). The method of static respirometry provides an average measurement of $\dot{M}O_2$ over the experimental period by comparing the initial oxygen concentration of the water with the final oxygen concentration. Consequently, this procedure does not allow for periods of activity and these measurements are at an intermediate state of metabolism, representing R_r. There may be substantial variation in R_r of lobsters due to alternation of behaviour between inactive to short periods of fully active swimming (Perera et al., 2005). Physiologists regularly make considerable effort to eliminate this source of variation in order to more accurately determine metabolic rates. Static respirometry also usually only permits short measurement periods during phyllosoma studies because it requires oxygen concentrations to be maintained above 70% (Ikeda et al., 2000) since $\dot{M}O_2$ in phyllosoma is reported to be dependent on oxygen saturation of ambient water (Belman and Childress, 1973).

Arguably the most useful measurement in intra- and inter-specific comparisons is that of R_s because it allows a standard reference of an unstressed animal for species comparisons (Radull et al., 2002). Static respirometry may be used to estimate R_s for inactive marine species under conditions of zero swimming activity (when activity levels are monitored) (Killen et al., 2007). However, for free-swimming zooplankton and active fish species, R_s can be determined by extrapolating

metabolic rates at different activity levels to zero activity using flow-through respirometers or swim tunnels (Brett, 1964; Halcrow and Boyd, 1967; Fry, 1971; Buskey, 1998; Ohlberger et al., 2007). Alternatively, intermittent flow-through respirometry eliminates problems associated with periods of activity and permits repeated measurements of $\dot{M}O_2$ during short time intervals over extended periods (Steffensen, 1989), which allows more accurate estimations of R_s.

For intermittent flow-through respirometry, R_s has been defined in several ways, from the average of the lowest two (Lahti et al., 2002) up to six (Weiser and Medgyesy, 1990) hourly oxygen consumption rates to the lowest 10% of all values (Herrmann and Enders, 2000; Fitzgibbon, 2010). An increase in $\dot{M}O_2$ is also commonly observed in respirometry studies due to transfer stress (Cech, 1990), requiring an acclimation period before metabolic rates become stable, which is referred to as the adaption phase (Herrmann and Enders, 2000). Therefore, intermittent flow-through respirometry provides further benefits including allowing adequate acclimation, avoiding accumulation of toxic excretory products, and preventing large fluctuations in in oxygen concentrations (Steffensen, 1989; Fitzgibbon, 2010). Intermittent flow-through respirometry has been shown to be an effective method for determining metabolic rates in juvenile and adult spiny lobsters (Crear and Forteath, 2000; Kemp et al., 2009) and puerulus (Fitzgibbon, 2010). However, this method of respirometry has not been used previously in phyllosoma due to their minute size and requirement for much smaller volume chambers. This was overcome by customising small diameter sections of acrylic tube and enabling oxygen probes to be inserted directly into chambers to reduce the volume of water required. The development of this method enabled precise measurements of phyllosoma metabolic rates in Chapter 3.

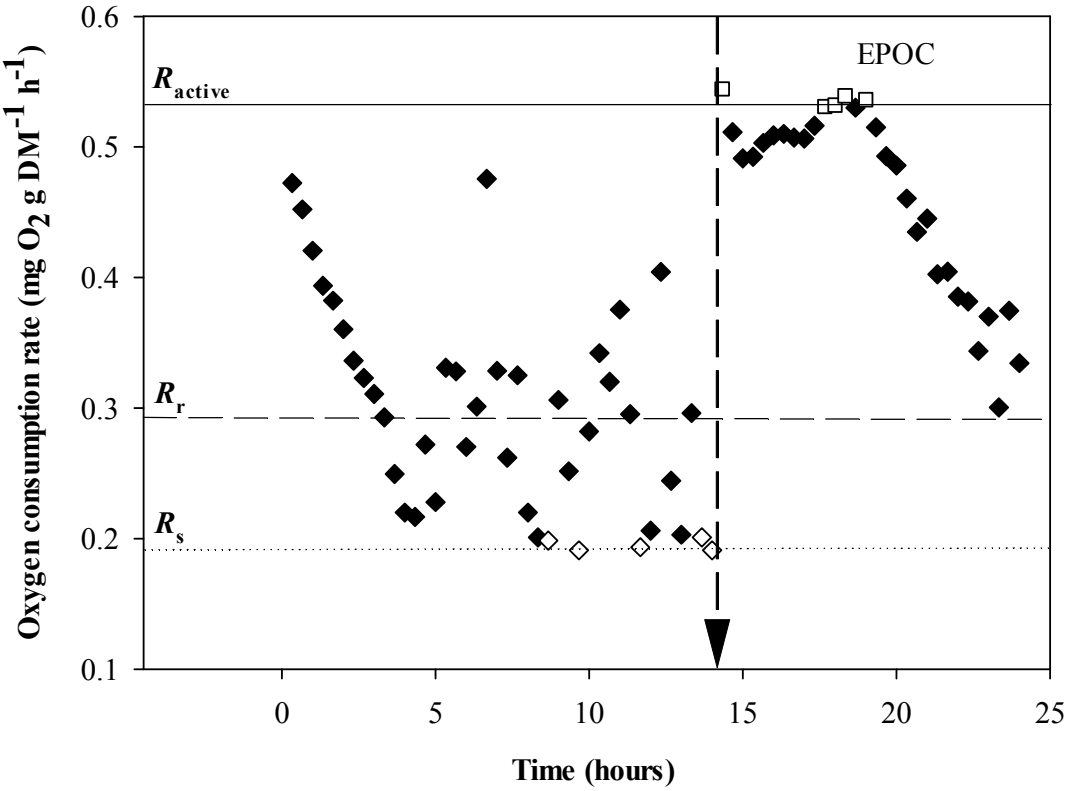

Figure 1.1. Oxygen consumption rate ($\dot{M}O_2$) measurements from an individual *Sagmariasus verreauxi* juvenile, which shows the definition of different metabolic states. The arrow indicates the time where the lobster was exercised to exhaustion using a chase protocol. The dotted horizontal line represents the standard metabolic rate (R_s) as the lowest 10% of $\dot{M}O_2$ measurements (◊) prior to the chase protocol. The dashed horizontal line represents routine metabolic rate (R_r) as the average of $\dot{M}O_2$ measurements prior to the chase protocol. The solid horizontal line represents active metabolic rate (R_{active}) as the highest 10% of $\dot{M}O_2$ measurements (□) following a chase protocol. Measurements of $\dot{M}O_2$ after the arrow represent excess post-exercise oxygen consumption (EPOC).

1.5.2 Aerobic scope

Aerobic scope is an important element of an animal's physiological characteristics for survival in their natural environment (Webb, 1986). Activity causes a large increase in $\dot{M}O_2$ above R_s levels (Crear and Forteath, 2001), which may be used to estimate the R_{active} of an animal (including lobsters). The aerobic scope for activity is defined as the difference between R_s and R_{active} (Korsmeyer et al., 2002). Elevated R_s have also been associated with a high aerobic capacity (Lewin, 1982), and may be related to an excessively large R_{active} and, therefore, large aerobic scope. Gaining information on the aerobic scope of an organism at various sizes or stages of development is important because aerobic scope represents the capacity to perform oxygen-consuming processes above the minimum metabolic requirements and compensate for physiological challenges (Djawdan et al., 1997; Bochdansky et al., 2005). However, information on the aerobic scope in crustaceans is limited due to the difficulties of accurately measuring R_s and R_{active} (Booth and McMahon, 1992). For example, many previous studies on crustaceans have typically under-estimated R_{active} due to not fully exerting the oxygen transport system during exercise (Booth and McMahon, 1992). Conversely, the intensive chase protocol employed in this research is effective in accurately measuring R_{active} and was used to define the aerobic scope and factorial aerobic scope (FAS; division of R_{active} from R_s) for juvenile *S. verreauxi* in Chapter 5 to determine their capacity to respond to stressors.

1.5.3 Body mass

The metabolic rate of an animal, like many other physiological processes, is related to body mass by an allometric relationship (Winberg, 1961; Schmidt-Nielsen, 1972; White and Seymour, 2005). The concept that the effect of body size on

metabolic rate might reflect the rate of heat production and the surface area over which it is dissipated ($M^{0.67}$) was first suggested by Sarrus and Ramaeux 1838 (cited by Brody, 1945). This was supported by Rubner (1883) who found that R_s of dogs was independent of mass when divided by surface area, which became known as Rubner's surface law of metabolism. This theory remained for nearly 50 years, until Kleiber's monograph (Kleiber, 1932; cited by Kleiber, 1961) found that metabolic rate was proportional to body mass raised to a mass scaling exponent (exponent used to describe the relationship between metabolic rate and body mass) significantly higher than 0.67, and a value of 0.75 was consequently accepted. The 0.75 scaling of R_s has been in widespread use for over 70 years. This exponent was supported by Brody's famous mouse-to-elephant curve (Brody, 1945), which included almost the largest order of magnitude in body mass possible in terrestrial mammals. However, 0.75 scaling lacked a common theoretical explanation until West et al. (1997) proposed that a unified mechanism underlies these laws; living things are sustained by the transport of materials through linear networks that branch to supply all parts of the organism. Despite this, the use of a universal mass scaling exponent for R_s has been questioned (Dodds et al., 2001; Bokma, 2004; Kozlowski and Konarzewski, 2004; White et al., 2006) and White et al. (2006) suggested the mass scaling exponent of fish is significantly higher than 0.75. It has also been proposed that this allometric relationship varies with development (Bokma, 2004; Burggren, 2005; Hunt von Herbing, 2005; Rombough, 2006), although it is not known whether the mass scaling exponent changes suddenly or gradually (Moran and Wells, 2007). Body mass also influences the ammonia-N excretion rate of crustaceans (see section 1.7). The effect of body mass on the metabolic rate and ammonia-N excretion rate of lobsters has not been studied previously through ontogeny, even though body mass is the main

endogenous factor influencing physiological processes, and was an aim of this thesis (Chapter 3).

1.5.4 Temperature

The natural distribution of marine organisms is strongly related to temperature because their thermal tolerance is closely matched to the temperature regime of their environment (Anger, 2001; Pörtner, 2001). *S. verreauxi* are a warm temperate lobster species that is distributed over a large latitudinal range (Montgomery and Craig, 2005), suggesting the species has a wide thermal tolerance. A mismatch between oxygen demand and the limited capacity of ventilatory and circulatory systems to supply oxygen to tissues is suggested to be the first mechanism setting the thermal tolerance limits of marine organisms (Frederich and Pörtner, 2000; Mark et al., 2002; Pörtner and Knust, 2007). However, beyond thermal tolerance limits, oxygen deficiency and physiological disturbances cause metabolic depression, which is characterised by a decrease in R_s (Melzner et al., 2006) and the onset of anaerobic metabolism (Frederich and Pörtner, 2000).

Although evidence of thermal tolerance limits in crustaceans have mainly focussed on adults (Frederich and Pörtner, 2000; Stillman, 2002; Lee, 2003; Fangue et al., 2006; Compton et al., 2007; Wittmann et al., 2008) and have rarely investigated different sized animals of the same species (Frederich and Pörtner, 2000; Melzner et al., 2006; Pörtner and Knust, 2007; Wittmann et al., 2008), water temperature has the potential to strongly affect the distribution of planktonic larvae and impact recruitment and settlement of juvenile lobsters. Studies on the thermal tolerance of larval and juvenile spiny lobsters are limited, despite the fact they may experience stronger temperature fluctuations and are more susceptible to thermal stress than adults (Anger et al., 2003). Studies on the effects of temperature on physiology can

help to understand the mechanisms setting the thermal tolerance limits of organisms and identify potential climate change effects on the species' distribution. Therefore the thermal tolerance of two different sizes of juvenile *S. verreauxi* was examined in Chapter 6 in order to identify potential climate effects on their distribution.

Temperature is a parameter that is easily manipulated in laboratory culture and has a major effect on growth, survival (Bermudes and Ritar, 2008), and rate of metabolic processes in crustaceans (Anger, 2001). An increase in temperature both reduces oxygen solubility in seawater and increases oxygen demand in crustaceans (Spicer, 1993). Following exposure to higher temperatures, an animal's metabolic rate increases, whereas when an animal is transferred to lower temperatures, the metabolic rate decreases (Precht, 1958). The effect of temperature on $\dot{M}O_2$ can be compared quantitatively among individuals, species, and developmental stages using the temperature coefficient Q_{10}. This index represents the ratio of two $\dot{M}O_2$ readings measured at two different temperatures. Most crustaceans have a Q_{10} between 2 and 3 within their thermal tolerance limits (Katsanevakis et al., 2007), while values approaching 8 indicate thermal sensitivity (Johnston et al., 1991). Many ectotherms display a typical stress response and increase in $\dot{M}O_2$ when they are exposed to an acute change in temperature (Bullock, 1955; Grainger, 1958; Prosser, 1973). It has also been suggested that temperature affects the development time and moulting frequency of lobsters (Kittaka, 1994) and may influence survival during metamorphosis to the puerulus stage (see Chapter 2). The effect of temperature on $\dot{M}O_2$ and metamorphosis of *S. verreauxi* phyllosoma during the final phyllosoma instar was examined in Chapter 2 to determine if a change in temperature is a cue for metamorphosis.

1.6 Biochemical composition

Changes in the ratios of certain chemical fractions of biomass have been frequently used as indicators of larval viability, which may be correlated with the overall chances of survival (Ferron and Leggett, 1994; Suthers, 1998). Studies on aquatic crustaceans have generally found three energy sources; lipids, proteins, and carbohydrates in the form of glucose and glycogen (Jeffs et al., 1999). Lipids have been reported to be the primary storage product in late stage phyllosoma, which are then used as an energy source during the non-feeding puerulus stage (Jeffs et al., 1999; 2001a; 2001b). Lipids also fuel almost all endergonic processes as well as maintaining the structural and physiological integrity of cellular and sub-cellular membranes (O'Connor and Gilbert, 1968). Proteins comprise most of the musculature, the epidermal and nervous tissues, and a large proportion of the cuticle (together with chitin) and are incorporated in hormones as well as being an important energy source in crustaceans (Lemos and Phan, 2001). The biomass of crustaceans is predominantly composed of protein (>30%), lipids (<20%), chitin (<15%), and only <5% are carbohydrates (Anger, 2001). Variations in individual biochemical components during development may indicate shifts in substrate utilisation (Johnston et al., 2004) and are crucial in identifying the components that are most important as energy reserves and those catabolised (Olsen, 1998).

Since the quantities of carbon (C) and nitrogen (N) are strongly associated with the amounts of total lipid and protein, respectively, elemental analysis may be used for indirect estimates of biochemical composition (Childress and Nygaard, 1974; Anger and Harms, 1990; Anger, 1998). Conversions of N to protein are commonly based on a conversion factor of 6.25 (Le Vay et al., 1993), which is derived from the average N content of proteins (16%), assuming all measured N is bound in this

fraction. Elemental analysis is commonly used for studies on wild larvae due to limited sample size. However, energy estimates from C and N data are less reliable than those based on analyses of biochemical composition (Anger, 2001). Despite Ikeda et al. (2011) measuring C:N ratios in *Panulirus ornatus* from hatch to the third last phyllosoma stage (instar 9 in this species), changes in C:N ratios and biochemical composition during the entire course of larval development have not been measured previously in spiny lobsters. Therefore, the biochemical and elemental composition of larvae from hatch to puerulus was analysed in response to stocking density in Chapter 4. Stocking density was used as a treatment parameter because it affects phyllosoma feed intake (Smith and Ritar, 2006) and therefore may also affect the ability of phyllosoma to accumulate the required energy reserves to survive and successfully complete metamorphosis.

1.7 Ammonia-N excretion rate

The nitrogenous end products that may be excreted by animals include ammonia, urea, uric acid, or amino acids, which all represent losses of chemical energy (Wright and Wood, 1985; Greenaway, 1991; Anger, 2001). In crustaceans, ammonia (NH_3) is the only major waste product (Anger, 2001) and the physiological mechanisms and pathways of ammonia excretion have been reviewed extensively by Greenaway (1999). Due to its toxicity, NH_3 cannot be stored but is excreted directly into the surrounding water, most commonly in the ionised form (NH_4^+) (Chapters 3, 5, and 6). The rate of N excretion in ammoniotelic animals is therefore usually measured in terms of ammonia-N released per unit time (Anger, 2001). Since successive developmental stages normally increase in biomass, the excretion rate per individual increases as a function of stage (Anger, 2001). In contrast, the mass-

specific excretion rate decreases with increasing biomass (Capuzzo and Lancaster, 1979; Sasaki et al., 1986; Agard, 1999).

Ammonia-N excretion in spiny lobsters, as in all crustaceans, is primarily via the gills into surrounding water, either by passive diffusion or actively against a concentration gradient through an exchange system involving NH_4^+ (Kormanik and Cameron, 1981). However, if respiratory function becomes limited or fails, lobsters are unable to excrete ammonia via the gills and ammonia is accumulated in the haemolymph (Huang and Chen, 2001). High levels of ammonia in the haemolymph are therefore usually indicative that ammonia-N excretion is impaired (Huang and Chen, 2001) and may be associated with stressful conditions such as aerial exposure, high and low salinities, or extreme temperatures (Vermeer, 1987; Chen et al., 1994; Schmitt and Uglow, 1997a; 1997b). Therefore, the effects of thermal stress on haemolymph ammonia concentrations were examined in juvenile lobsters in Chapter 6 to determine if ammonia is accumulated in the haemolymph at extreme temperatures. Ammonia-N excretion rates of phyllosoma were also examined in response to stocking density (Chapter 3) and handling, anaesthesia, activity (Chapter 5), and temperature (Chapter 6) in juvenile lobsters to determine how energy substrate utilisation is influenced by each of these conditions through the use of the O:N ratio.

1.8 The O:N ratio

The proportion of oxygen (O) respired to N excreted is widely accepted as an indicator of the metabolic substrate for energy production (Capuzzo and Lancaster, 1979; Chen and Lai, 1993; Chu and Ovsianico-Koulikowsky, 1994; Agard, 1999; Ikeda et al., 2011). This is due to the predictable stoichiometric relationship between O and N in a generalised protein. Variability in the O:N ratio occurs mainly as a result of changes in the rate of N excretion, while oxygen consumption tends to

remain reasonably constant (Anger, 2001). Therefore, this index varies mainly with the N content in the diet, representing the biochemical composition of utilised energy reserves originating from either larval biomass or food (Ikeda, 1974; Mayzaud and Conover, 1988). Low O:N ratios are an indication that protein is being used as an energy source, and conversely, the higher the O:N ratio, the more carbohydrates or lipids are being utilised (Corner and Cowey, 1968). According to the average elemental composition of the main compound classes, an atomic O:N ratio with a minimum value of 7 indicates pure protein catabolism, while a ratio of about 24 is usually an indication that equal amounts of protein and lipid are being utilised (Brafield and Solomon, 1972; Ikeda, 1974). Since the amount of carbohydrates within the biomass of most crustaceans is insignificant, variations in the O:N ratio primarily reflects changes in the proportions of protein and lipid degeneration (Anger, 2001). In various crustacean larvae, consistently low O:N ratios have been observed, indicating protein-based metabolism (Anger et al., 1989; Chu and Ovsianico-Koulikowsky, 1994; Anger, 2001), which has often been associated with stressful conditions (Pillai and Diwan, 2002). Protein metabolism is also less efficient than lipid/carbohydrate metabolism. Therefore, O:N ratios approaching 7 should be avoided in an aquaculture situation because protein for energy will be highly uneconomical (Pillai and Diwan, 2002). The O:N ratio was evaluated for phyllosoma in Chapter 3 and for juveniles in Chapters 5 and 6.

1.9 Stress

Stress is a general term which indicates a change in physiological condition in response to internal or external stimuli (stressors) beyond the normal physiological range (Chrousos and Gold, 1992; Bonga, 1997; Taylor et al., 1997; Barton, 2002). Compensatory mechanisms are evolved from dealing with stressors encountered in

the wild such as bursts of activity to escape from predators (Taylor et al., 1997). Stress is also a regular occurrence in crustacean aquaculture, and potential stressors include handling, activity associated with escaping capture, transport, drug administering, and confinement (Iversen and Eliassen, 2009). Stress can either be reversible, if the physiological disruption is within the homeostatic capability of the lobster, or it can be irreversible and eventually lead to death (Taylor et al., 1997). Reversible stressors create physiological responses that threaten or disrupt the homeostatic equilibrium and result in a sequence of behavioural and physiological responses considered to be compensatory or adaptive, permitting the animal to overcome the stress (Iversen and Eliassen, 2009). In the case of chronic stress, animals may lose their adaptive ability and become dysfunctional, which may cause reduced growth, reproductive capacity and resistance to pathogens. Stress itself cannot be measured directly; however, physiological changes associated with stress responses can be evaluated quantitatively by changes in $\dot{M}O_2$ to determine which stimuli are stressful (Lorenzon et al., 2007). Other indicators of stress used in crustaceans include accumulation of haemolymph lactate, glucose, and ammonia and reduced pH (Taylor et al., 1997). Stress in spiny lobsters may considerably reduce survival in culture or during transport (Taylor et al., 1997). Therefore, changes in $\dot{M}O_2$ of *S. verreauxi* juveniles were examined in Chapter 5 to assess the metabolic stress responses to handling, force feeding, anaesthesia and activity. Changes in haemolymph parameters were also used to determine responses of *S. verreauxi* juveniles to temperature stress in Chapter 6.

1.10 Metamorphosis

There is a critical process at the end of larval development, which separates the final instar phyllosoma from the puerulus stage and is known as metamorphosis.

The location and trigger for metamorphosis likely influences the distance that non-feeding pueruli must travel to reach inshore settlement sites. If these travel distances are extensive, this may exhaust the limited energy reserves of pueruli and therefore reduce recruitment (Lemmens, 1994; McWilliam and Phillips, 1997; Jeffs et al., 1999; Jeffs et al., 2001a). Several researchers have suggested that metamorphosis of the final phyllosoma is triggered by an environmental cue associated with the slope region close to the shelf-break, such as lower salinity and higher productivity (Burke, 1983; Phillips and McWilliam, 1986; Booth and Phillips, 1994; McWilliam and Phillips, 1997). Other researchers suggested metamorphosis is induced by specific physical or chemical cues associated with the adult habitat and in the absence of such cues, larvae may delay their metamorphosis, within certain limits, to search for a more suitable habitat (Pechenik, 1990; Zimmerfaust and Tamburri, 1994; Pechenik, 1999).

In culture, metamorphosis of final stage phyllosoma of spiny lobsters has occurred in the absence of any of the possible natural cues (Jeffs et al., 2001b). Kittaka (1994) observed that metamorphosis occurred after *S. verreauxi* phyllosoma had developed through 17 instars, which was mainly influenced by a combination of water temperature and feeding rate, and is dependent upon the nutritional condition of later stage larvae. Therefore, it is believed that since the puerulus is a non-feeding stage, metamorphosis may result from the culmination of sustained nutrition and energy reserves accumulated through the later larval stages and may only occur after the final stage phyllosoma has reached a critical, specific, level of stored energy reserves (McConaugha, 1982; McWilliam and Phillips, 1997). Final stage phyllosoma are physiologically capable of responding to metamorphosis cues, but in the absence of such cues many larvae do not progress toward metamorphosis (Forward et al., 2001). Hence, metamorphosis is more likely to be triggered by a

combination of exogenous cues rather than just an internal energy storage threshold (Jeffs et al., 2001a). Therefore, the effect of temperature change during the final instar of *S. verreauxi* was examined in Chapter 2 to determine if temperature is a cue for metamorphosis. Temperature was examined as a possible cue for metamorphosis due to the ability of this exogenous factor to affect growth and development of phyllosoma (Kittaka, 1994) and because of the change in temperature preference of *S. verreauxi* approaching metamorphosis (Kittaka et al., 1997).

1.11 Structure of the thesis

The thesis contains a general introduction, five research chapters (Chapters 2-6), and a general discussion incorporating data from the research chapters and combining this with additional data from different sized juvenile lobsters. Each research chapter has been structured as a journal manuscript and it is the author's intention to publish this information in scientific journals following examination of the thesis. Consequently, some of the content in the thesis may be repeated, in particular in the materials and methods and results.

Chapter 2 was a preliminary study designed to justify a more comprehensive investigation of the physiological factors affecting larval development leading up to metamorphosis that were examined in Chapters 3 and 4. Chapters 3 and 4 used the same hatch of animals and growth data and examined the physiological effect of culture density on larvae from hatch to puerulus.

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EFFECTS OF TEMPERATURE ON ROUTINE METABOLIC RATE, INSTAR DURATION, AND METAMORPHOSIS OF FINAL INSTAR *Sagmariasus verreauxi* PHYLLOSOMA

Chapter II

2.1 Abstract

Planktonic phyllosoma metamorphose to the non-feeding nektonic puerulus stage, which swim across the continental shelf towards the coast to settle and moult to the benthic juvenile stage. The process of metamorphosis is thought to be triggered by chemical or environmental cues, but is still poorly understood. Potential cues for metamorphosis may influence the distance pueruli need to swim to reach inshore settlement areas. This study examined the effects of temperature on the routine metabolic rate (R_r), instar duration, and metamorphosis success of the spiny lobster *Sagmariasus verreauxi* during the final phyllosoma instar (instar 17) to assess the probability that temperature is a cue for the onset of metamorphosis. Phyllosoma were reared at 23°C and halfway through the final instar (Day 12) temperature was decreased to 19°C, maintained at 23°C or increased to 25°C. The R_r of phyllosoma significantly decreased and remained low after the temperature was reduced to 19°C but was not significantly different at 25°C or 23°C. The metabolic responses to a change in water temperature were typical of thermal acclimation at all study temperatures and indicated these temperatures are within the thermal tolerance range of instar 17 *S. verreauxi* phyllosoma. Phyllosoma held at 19°C stopped feeding five days before metamorphosis and at least five days earlier than those at the other temperatures. This difference in feeding indicated the reduced metabolism and energy requirements of phyllosoma at lower temperatures. Temperature change did not significantly affect the duration of the final instar, which ranged between 28.0 ± 1.11 d to 30.5 ± 2.19 d, suggesting that it is not a cue for metamorphosis. Survival of phyllosoma leading up to metamorphosis, however, was lower at 19°C (43%) and 25°C (45%) than at 23°C (52%). Of the surviving larvae that attempted

metamorphosis, significantly more phyllosoma completed metamorphosis and progressed to the puerulus stage at 19°C (75%) and 23°C (33%) than at 25°C (0%), suggesting warmer temperatures inhibit metamorphosis. Although water temperature was not identified as a possible cue for metamorphosis, the results of this study indicate that culture temperatures of 23°C and 25°C are not suitable for final stage *S. verreauxi* during metamorphosis and a temperature closer to 19°C is recommended. This suggests environmental water temperature may influence the patterns of puerulus settlement and subsequent recruitment of juveniles to coastal regions and associated valuable fisheries.

2.2 Introduction

Reduced recruitment, increasing global demand, a high market value, and continued depletion of wild stocks has generated substantial interest in lobster propagation in Australia and internationally (Jeffs and Hooker, 2000; Linnane et al., 2010). One of the most important developments towards commercial production of spiny lobsters, such as *Sagmariasus verreauxi*, is to define their environmental requirements in order for phyllosoma to successfully progress through metamorphosis.

The lifecycle of spiny lobsters includes a transition from the oceanic planktonic phyllosoma phase to a nektonic puerulus through the process of metamorphosis (Gurney, 1936; Lesser; 1978; Jeffs et al., 2005). The puerulus is a short-lived, non-feeding stage that resembles adults in shape and swims across the continental shelf towards the coast, where it settles in shallow waters and then moults to the benthic juvenile stage (Menzies and Kerrigan, 1979; Phillips; 1981; Booth and Ovenden, 2000). In many larval decapods, the process of metamorphosis is triggered by specific chemical or environmental factors, which are referred to as cues (Crisp, 1974; Gebauer et al., 2003). Larvae will also only survive and successfully complete metamorphosis if they are competent, which is defined as the phase where larvae are physiologically and morphologically prepared to respond to cues that trigger metamorphosis (Crisp, 1974; Pawlik, 1992; Avila, 1998; Bryan et al., 1998; Pechenik, 1999; Gebauer et al., 2003). However, the physiology of lobsters during metamorphosis is poorly understood (Jeffs et al., 2005). There is limited information on the location and trigger for metamorphosis of the final instar phyllosoma (instar 17 in *S. verreauxi*) to puerulus due to the difficulties in obtaining direct observational or

experimental evidence (Cobb, 1997; McWilliam and Phillips, 1997; Phillips and Pearce, 1997; Chiswell and Booth, 2005; Jeffs et al, 2005; Phillips and McWilliam, 2009). Identifying potential environmental cues for metamorphosis is important because many larvae do not progress toward metamorphosis if cues are absent (Forward et al., 2001) and there is a "delay of metamorphosis" (Crisp, 1984). Delay of metamorphosis is believed to have drastic ecological and physiological costs because it delays settlement to a new benthic environment (Forward et al., 2001) and prolongs the utilisation of energy reserves and reduces the chances of finding a suitable habitat. Delay of metamorphosis may also reduce post-metamorphic size and growth (Pechenik et al., 1993).

Limits of thermal tolerance and optimal temperatures for culture of different larval stages may be estimated from observations in the wild (Bermudes and Ritar, 2004). *S. verreauxi* phyllosoma are transported horizontally by currents and undergo large diurnal vertical movements in the wild (Booth, 1986). Relatively small fluctuations in temperature of up to 4°C were reported for *Panulirus cygnus* phyllosoma during diurnal vertical migrations (Rimmer and Phillips, 1979). Phyllosoma are therefore clearly able to survive temperatures within this range. Under laboratory conditions, a culture temperature of 21 to 23°C is suitable for early stage *S. verreauxi* phyllosoma, but this temperature may be too high for later stage phyllosoma approaching metamorphosis. Kittaka et al. (1997) previously reduced the water temperature to 20°C for mid- and late-stage *S. verreauxi*, which improved survival from hatch to puerulus. A downward shift from 26°C to 24°C also produces optimal growth and survival for late stage *Panulirus japonicus* (Matsuda and Yamakawa, 1997).

Temperature affects the ability of a marine animal to survive in a particular environment, and also has significant effects on the rate of individual physiological functions, such as metabolic rate, growth and development. Newell and Branch (1980) recognised three distinct time phases in response to a change in environmental temperature. Firstly, there is an acute response in physiological functions, which is usually characterised by metabolic rate exceeding normal values followed by a stabilisation period over time. Secondly, "thermal acclimation" follows long-term exposure to a change in temperature and the animal may adjust the rate of physiological functions. Lastly, a prolonged change in environmental temperature may, over many generations, favour genotypes that are adapted to the new temperature. There is no information on the ability of phyllosoma to acclimate to an acute change in temperature and its effect on physiological processes, such as metabolism, that influence larval development. Despite this, variations in metabolic rate associated with changes in temperature have been reported for several other crustacean species (Burton et al., 1981; Cockcroft and Wooldridge, 1985; Bermudes and Ritar, 2004; Haukenes et al., 2009). Most of these crustaceans are able to cope with some level of temperature change by adjusting their metabolic rate (Burton et al., 1981). However, as the temperature shifts further from the optimum level a critical point is reached where the metabolic rate can no longer be sustained and death is imminent.

Oxygen consumption in crustaceans, as in other ectotherms, increases with temperature (Cockcroft and Wooldridge, 1985). An increase in feed intake with temperature also accelerates the rate of development in phyllosoma (Tong et al., 2000) and reduces the moult increment (Anger, 2001). However, increased feed intake at elevated temperatures is often weaker than the concurrent acceleration of

development leading to a reduction in the cumulative amounts of food that are ingested within a particular instar (Anger, 2001) and phyllosoma requiring more energy to maintain metabolism at higher temperatures (Matsuda and Yamakawa, 1997). Conversely, at lower temperatures the feeding activity generally decreases with decreasing metabolic requirements (Anger, 2001). A drop in water temperature may therefore cause a reduction in metabolic rate and allow phyllosoma to accumulate the reserves required for development to puerulus (Moss et al., 2001). Knowledge about a change in water temperature may also provide valuable information on a critical environmental cue for metamorphosis in culture.

Defining where metamorphosis occurs in the wild and what triggers this process in final stage phyllosoma is crucial to understanding the recruitment processes of any spiny lobster species (Cobb, 1997). Information on the distribution of phyllosoma in coastal waters has prompted several researchers to suggest that metamorphosis of the final stage phyllosoma is a response to stimuli triggered by an environmental cue that exists in waters just beyond the continental shelf. Phillips and McWilliam (1986) suggested that a change in salinity near the shelf-break might be a possible cue. However, numerous chemical and physical factors were examined as possible cues for metamorphosis in *P. cygnus* and other palinurid species, with no evidence to support these theories (Phillips and Pearce, 1997). Determining whether metamorphosis is influenced by cues during culture is also important. In culture, metamorphosis of final-stage phyllosoma of spiny lobsters, including *S. verreauxi*, has occurred without introducing specific triggers (Jeffs et al., 2001). Kittaka (1994) observed that metamorphosis by *S. verreauxi* only occurred after phyllosoma had developed through 17 instars and was influenced mainly by a combination of water temperature and feeding rate. However, many cultured phyllosoma die just before,

during or just after moulting or metamorphosis (Matsuda and Yamakawa, 1997). Metamorphosis may therefore be dependent upon the nutritional condition of later stage larvae (Kittaka, 1994). Because the puerulus is a non-feeding stage, it is believed metamorphosis will only occur when the final stage phyllosoma reaches a critical level of stored energy reserves and becomes competent (McWilliam and Phillips, 1997).

The aims of the present study were firstly, to determine whether a change in temperature is a cue for metamorphosis in *S. verreauxi* by measuring the duration of the final phyllosoma instar; second, determine the effect of a change in temperature on the ability of phyllosoma to complete metamorphosis successfully; lastly, to determine the physiological response to a change in water temperature during the final instar by acute scale measurements of phyllosoma metabolism. This will provide valuable information on physiological responses to a change in temperature and the ability of phyllosoma to acclimate to different temperatures.

2.3 Materials and methods

2.3.1 Experimental animals

Broodstock were held in captivity in a 4,000 l fibreglass tank year round under a regime of ambient photoperiod and water temperature (11°C-19°C), 33-35 psu salinity, pH approximately 8.1, and 90-100% oxygen saturation at the Institute for Marine and Antarctic Studies (IMAS), Taroona, Hobart. Phyllosoma were hatched in February from female lobsters weighing approximately 1.5 kg and then reared at 21-23°C in 200 l cylindrical tanks with a flow rate of 3 exchanges h^{-1} on ambient photoperiod and fed *Artemia* and mussel gonad to the second last instar (instar 16). Phyllosoma were then haphazardly selected from mass culture tanks and measured for total length (TL) and carapace width (CW) using a profile projector (Nikon 6C, Japan), then staged under a dissecting microscope (AIS Optical) at 10-40x magnification according to Kittaka et al. (1997), and assessed for limb damage using a Limb Damage Index (LDI) from 1 to 5 where: 1 indicated all limbs intact; 2 indicated one limb missing; 3 indicated two limbs missing; 4 indicated three limbs missing; and 5 indicated more than three limbs missing. For each animal, the specific position and fraction of damaged limbs remaining was recorded.

Three temperature treatments of 19ºC, 23ºC, and 25ºC were chosen so there were comparisons for a temperature lower than the 20 ºC previously used by Kittaka et al. (1997) (19ºC); a temperature at which mid-stage phyllosoma were reared (23ºC) and; a temperature higher than that used for rearing mid-stage phyllosoma (25ºC). Instar 16 phyllosoma were randomly distributed into the three temperature treatments and individually stocked in 1.6 l cylindrical containers with a flow rate of 5 exchanges h^{-1} for daily observations of moulting. For each temperature treatment, phyllosoma

were also randomly distributed into two treatment groups (Groups A and B). Group A contained animals for examination of instar duration and metamorphosis success and Group B animals were used for measurements of routine metabolic rate (R_r). Eighteen phyllosoma (six phyllosoma at each temperature) in Group A and fifteen phyllosoma (five phyllosoma at each temperature) in Group B were initially individually stocked (total of 33 containers). Phyllosoma were immediately replaced with individuals of the same age if mortality occurred prior to temperature changes (see table 2.1).

2.3.2 Experimental systems and husbandry

The experimental system received seawater filtered to 1 µm and treated with ozone and ultra-violet irradiation according to Jensen et al. (2011) and consisted of two 50 l reservoir tubs where treated seawater was heated from 19ºC to either 23ºC or 25ºC using 2.4 kW heaters (model 525-2935, Istra Elements and Engineering Pty. Ltd., Caringbah, NSW). Water was pumped using aquarium pumps (Quiet1one 800, Aquasonic, Wauchope, NSW) to 1.6 l cylindrical plastic containers and delivered through a 180º nozzle positioned at the bottom centre of the containers to provide the required flow to assist phyllosoma suspension in the water column. The single pass flow of water to each container provided five exchanges h^{-1} (130 ml min^{-1}) and the volume was maintained at a constant 1.6 l with excess water exiting through a 700 µm screen positioned on the side of the container. Containers were cleaned (with detergent and hot water) once each fortnight and phyllosoma were transferred back to the clean containers. Phyllosoma were fed a combination of 0.1 ml of freshly prepared mussel gonad (~ 5 mm diameter pieces) daily and on-grown *Artemia* of approximately 8.0 mm in length three times each week.

Artemia cysts (AAA grade, Artemia Systems, INVE, Belgium) were hatched in 50 l conical tanks at 28°C in 1 µm-filtered and ozonated seawater with vigorous aeration and a 150-W incandescent light bulb suspended 0.5 m above the water surface. Hatched nauplii were rinsed in freshwater for 5 min and stocked in 670 l tanks at 5 *Artemia* ml^{-1} before on-growing to approximately 8.0 mm in flow-through tanks receiving a diet of blended brine shrimp food (consisting of rice pollard, soyflour and wheat flour; Eyre Peninsula Aquafeeds Pty Ltd, South Australia) and algae (T. *Isochrysis* and *Chaetoceros muelleri*). Prior to feeding to phyllosoma, gut bacteria in *Artemia* were minimised by purging in dense cultures of *C. muelleri* (10^7 cells ml^{-1}), as described by Tolomei et al. (2004), and 400 ppm formalin for 30 min before rinsing with filtered seawater and feeding to phyllosoma at a density of 20 *Artemia* l^{-1}. Uneaten *Artemia* remaining from the previous meal were flushed from experimental containers prior to feeding the next meal of *Artemia*.

Meals of fresh pieces (5.0 mm in diameter) of blue mussel (*M. edulis*) were fed to phyllosoma at a rate of 0.1 ml $phyllosoma^{-1}$. Uneaten mussel was siphoned from culture vessels each day before feeding the next meal of mussel.

2.3.3 Temperature treatment

The temperature was adjusted by disconnecting the 23°C incoming water line and connecting container inlets to either a 19°C or 25°C water line halfway through the final instar stage (Day 12). The average length of the final instar (in days) was based on previous laboratory observations by Kittaka et al. (1997) (23 d at 20°C). All treatments started at 23°C and 12 d after an animal had moulted to the final instar, temperatures were adjusted to either 19°C or 25°C, while 23°C was used as the control temperature. As each container had a flow of five exchanges h^{-1}, adjusted temperatures were achieved after approximately 12 min. The number of food

particles that remained before the next meal were recorded daily to assess feeding activity and the number of days phyllosoma stopped feeding before metamorphosis. Temperature treatments were maintained until all phyllosoma either completed metamorphosis or died. After this time, the average moult increment for final instar phyllosoma at each temperature was calculated as the time between moulting to instar 17 and metamorphosis. The average moult increment was used, in conjunction with metamorphosis success, to determine if temperature change during the final instar is a cue for metamorphosis or affects the ability of phyllosoma to complete metamorphosis.

2.3.4 Respirometry

Routine metabolic rate was measured by static respirometry. Chambers for respirometry were constructed from 50 ml syringes. Individual phyllosoma were stocked into 50 ml chambers covered with mesh (2000 μm) and immersed in an ambient tank (temperature bath) and left overnight to clear the digestive tract of food and faeces and allow the animal to acclimate to the chamber. Chambers were then sealed at 40 ml using the syringe plunger and left for 20 min before a 10 ml water sample from the chamber was delivered into a glass vial (leaving 30 ml) to determine initial dissolved oxygen (mg l^{-1}) using a luminescent dissolved oxygen optode (Hach LDO, HQ10). After a further 1 h, the final dissolved oxygen in the chambers was sampled. This process was replicated at the same time for a control chamber (without an animal), which was sampled simultaneously to measure background oxygen consumption ($\dot{M}O_2$). The R_r of phyllosoma was expressed in mg O_2 g DM^{-1} h^{-1} after the subtraction of background $\dot{M}O_2$ obtained from control chambers.

Routine metabolic rate was measured once on Day 10 (i.e. 10 d after phyllosoma moulted to instar 17), two days before the water temperature was changed

to the treatment temperatures (19°C, 23°C and 25°C). Routine metabolic rate was measured a further three times following the temperature change on Day 12; at 30 min, 7 h and 30 h after the temperature change. The phyllosoma were held in mesh covered chambers with flow-through seawater (5 exchanges h^{-1}) between each measurement. Following measurements of R_r, the TL, CW, instar stage, and dry mass (DM) of phyllosoma was measured. The DM was determined by rinsing phyllosoma in 0.5M ammonium formate to remove any salt and drying in an oven for 24 h at 60°C. The mass of each sample was measured to the nearest 10 µg on a precision balance (Mettler AT261 DeltaRange, Metler-Toledo, Switzerland).

2.3.5 Data analysis

Residual plots were used to explore normality and homogeneity of data. The number of individuals that progressed through metamorphosis within each temperature treatment and the time that elapsed from the first individual to the last individual event of metamorphosis were used as indicators of metamorphosis success. The number of individuals that successfully progressed to pueruli was plotted for each temperature treatment and analysed using a chi-square test. The mean instar duration at each temperature was analysed using a one-way ANOVA and Tukey's HSD tests for post-hoc multiple comparisons. Mean R_r measurements for temperature treatments over different time periods were analysed using two-way ANOVA and Tukey's HSD tests for post-hoc multiple comparisons. The level of significance for all analyses was determined at $P<0.05$. Data are presented as mean ± standard error (SE) unless stated otherwise. Statistical analyses were performed using SPSS version 16.0 (2007 SPSS Inc.).

2.4 Results

The mean duration of instar 17 was not significantly different between temperatures (one-way ANOVA; F= 0.390, df 2, 13, *P*= 0.685, Fig. 2.1) and ranged between 28.0 ± 2.27 and 30.5 ± 2.19 d at 23°C and 25°C, respectively. However, the number of phyllosoma that metamorphosed successfully to the puerulus stage was significantly different between temperature treatments (X^2= 6.303, df 2, *P*= 0.043). None of the 6 phyllosoma cultured at 25°C (0%) metamorphosed to puerulus successfully, compared to 3 from 4 (75%) cultured at 19°C (Fig. 2.2). Only 2 from 6 (33%) phyllosoma cultured at 23°C metamorphosed successfully to the puerulus stage. Phyllosoma in the 19°C treatment stopped feeding at least five days prior to metamorphosis compared to phyllosoma in the 23°C (0.8 d) and 25°C (1.0 d) treatments (one-way ANOVA; F= 6.669, df 2, 15, *P*= 0.01, Table 2.1).

Temperature did not have a significant effect on the survival of instar 17 phyllosoma (X^2= 0.100, df 2, *P*= 0.951, Table 2.1). For the surviving larvae, the TL (one-way ANOVA; F= 0.552, df 2, 29, *P*= 0.582), CW (one-way ANOVA; F= 0.155, df 2, 28, *P*= 0.857), and DM (one-way ANOVA; F= 2.039, df 2, 12, *P*= 0.181) of phyllosoma were not significantly different between temperature treatments. The TL (one-way ANOVA; F= 0.285, df 2, 14, *P*= 0.757), CW (one-way ANOVA; F= 1.887, df 2, 11, *P*= 0.207), or DM (one-way ANOVA; F= 0.012, df 1, 5, *P*= 0.920) of pueruli were also not significantly different between temperature treatments.

Table 2.1. Details of *Sagmariasus verreauxi* phyllosoma and puerulus size, condition and survival.

	19°C	**23°C**	**25°C**
Phyllosoma initially stocked[1]	11	11	11
Total length (mm) (±SE)	33.2 ± 0.54	32.8 ± 0.62	33.9 ± 0.48
Carapace width (mm) (±SE)	17.5 ± 0.22	17.4 ± 0.36	17.7 ± 0.31
Dry mass (mg) (±SE)	66.44 ± 7.35	57.03 ± 3.30	70.54 ± 3.47
Limb Damage Index (LDI)	2 ± 0.37	2 ± 0.31	2 ± 0.45
Mortality before metamorphosis[2]	13	9	13
Total phyllosoma stocked[3]	21	19	23
Phyllosoma survival (%)	43	52	45
Numbers to metamorphosis[4]	4	6	6
Days feeding stopped before metamorphosis[5] (± SE)	5.0 ± 1.58[a]	0.8 ± 0.48[b]	1.0 ± 0.63[b]
Number of mortalities during metamorphosis[6]	1	4	6
Number of live puerulus[7]	3	2	0
Puerulus total length (mm) (±SE)	23.1 ± 0.85	23.9 ± 1.35	24.9 ± 2.17
Puerulus carapace width (mm) (±SE)	5.7 ± 0.15	8.6 ± 1.61	9.1 ± 1.98
Puerulus dry mass (mg) (±SE)	54.54 ± 5.78	57.53 ± 2.19	52.66 ± 0.00

[1]Number of phyllosoma that were initially stocked at the start of the experiment; [2]Mortality of phyllosoma during instar 17; [3]Total phyllosoma that were stocked by the end of the experiment due to mortality; [4]Phyllosoma alive immediately prior to metamorphosis; [5]Number of days phyllosoma stopped feeding prior to metamorphosis; [6]Mortality during metamorphosis; [7]Numbers of live puerulus at each temperature. Letter superscripts indicate significant differences (P<0.05).

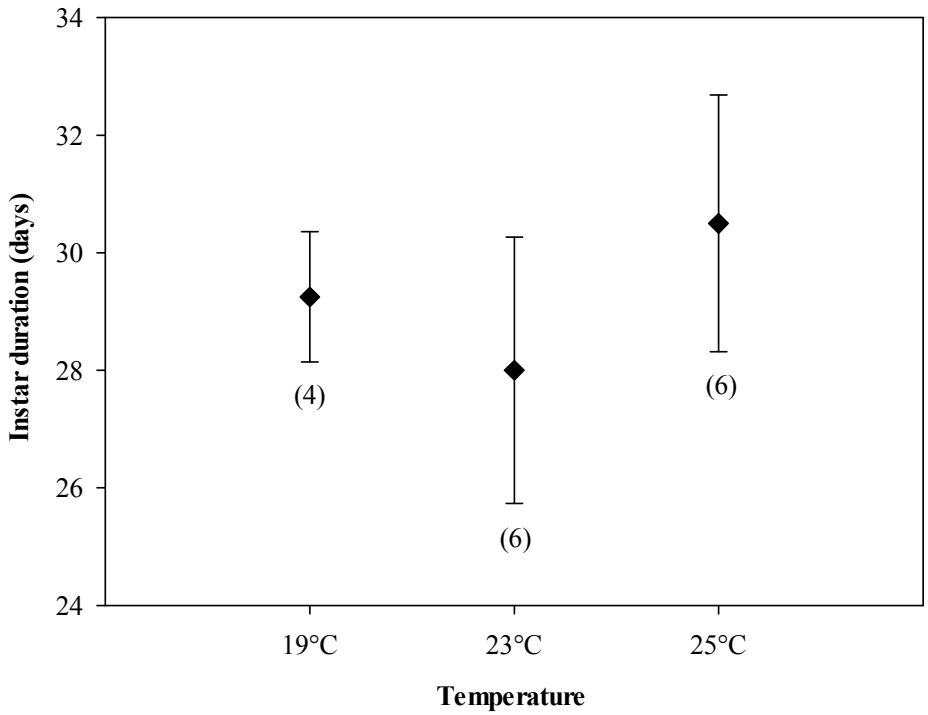

Figure 2.1. Duration (days) of instar 17 in *Sagmariasus verreauxi* phyllosoma cultured at 19°C, 23°C, and 25°C. Numbers in brackets represent the number of replicates in each treatment. Values are mean (±SE).

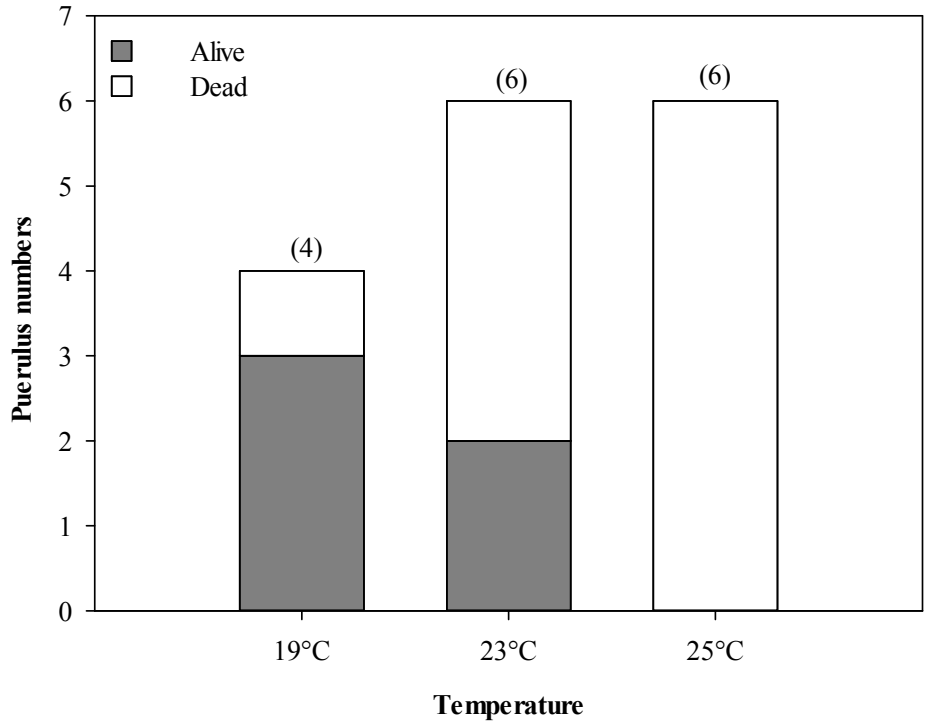

Figure 2.2. Metamorphosis success, in terms of number of puerulus alive after completing metamorphosis, of *Sagmariasus verreauxi* phyllosoma cultured at 19°C, 23°C, and 25°C. Numbers in brackets represent the number of replicates in each treatment.

The R_r of phyllosoma at 23°C (two-way ANOVA; F= 1.694, df 3, 12, *P*= 0.362) and 25°C (two-way ANOVA; F= 0.866, df 3, 12, *P*= 0.485) was not significantly affected by sample time (Fig. 2.3). However, R_r of phyllosoma was significantly lower when measured 1 h after the temperature was changed and remained low for 30 h in the 19°C treatment (two-way ANOVA; F= 6.569, df 3, 12, *P*= 0.007). At a sample time of -48 h the R_r was at least 20% higher compared to all other 19°C sample times. Routine metabolic rate at -48 h was not compared between temperature treatments because all these measurements were taken at 23°C and, therefore, data were pooled.

At 1 h after the temperature was changed, the R_r of instar 17 phyllosoma was highest for phyllosoma in the 25°C treatment (0.63 mg O_2 g DM^{-1} h^{-1}), which was nearly 50% higher than for the 19°C treatment (0.44 mg O_2 g DM^{-1} h^{-1}) at the same sample time. However, this was not significantly different (one-way ANOVA; F= 1.710, df 2, 9, *P*= 0.235). After 7 h, the R_r was not significantly different between temperature treatments (one-way ANOVA; F= 3.515, df 2, 9, *P*= 0.074) and was the same for phyllosoma in the 23°C (0.56 mg O_2 g DM^{-1} h^{-1}) and 25°C (0.56 mg O_2 g DM^{-1} h^{-1}) treatments, but was again lowest for phyllosoma held at 19°C (0.44 mg O_2 g DM^{-1} h^{-1}). This trend continued, with no significant difference in R_r between temperatures at the sample time of 30 h after the temperature change (one-way ANOVA; F= 1.329, df 2, 9, *P*= 0.312), where phyllosoma from the 25°C (0.54 mg O_2 g DM^{-1} h^{-1}) treatment recorded the highest R_r, and phyllosoma from the 19°C (0.45 mg O_2 g DM^{-1} h^{-1}) treatment, the lowest.

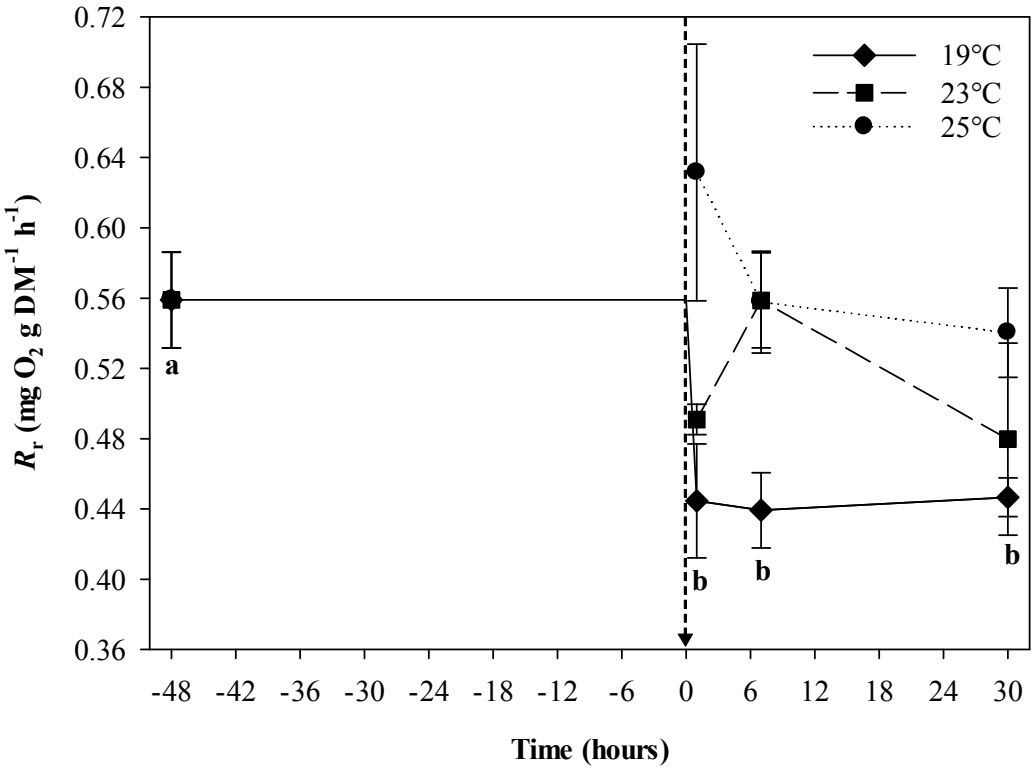

Figure 2.3. Routine metabolic rate (R_r) of instar 17 *Sagmariasus verreauxi* phyllosoma cultured at 23°C and 1 h, 7 h, and 30 h after the temperature was changed to 19°C or 25°C or maintained at 23°C. The arrow indicates the time in relation to when the temperatures were changed at 0 h. n= 12 at -48 h and n= 4 for all other data points. Data points bearing different superscripts are significantly different (P<0.05). Values are mean (±SE).

2.5 Discussion

2.5.1 Survival

Changing the water temperature mid-way through the final phyllosoma instar had a considerable impact on metamorphosis success and survival of *S. verreauxi* puerulus. The survival of phyllosoma normally decreases as the lower and upper bounds of their temperature tolerance range are approached (Bermudes and Ritar, 2008). All phyllosoma died during metamorphosis at 25°C which indicated that instar 17 phyllosoma in the present study had reached their upper temperature tolerance limit for successful metamorphosis. This does not suggest that phyllosoma would not be able to survive or acclimate to this temperature in previous instars, but they cannot survive metamorphosis at 25°C when reared at 23°C. Possible reasons for mortality at high temperatures include insufficient energy to meet the demands of metamorphosis, insufficient oxygen supply due to respiratory failure, the disturbance of cellular membrane structure and function (Willmer et al., 2000), or a combination of these factors around the time of a moult or metamorphosis (Bermudes and Ritar, 2008). Insufficient oxygen supply is also associated with a reduction in aerobic scope and is commonly used to determine the lower and upper temperature tolerance limits of marine organisms (Pörtner et al., 2005). Hatchery rearing of early stage *S. verreauxi* phyllosoma has previously been successfully conducted at 23°C (Moss et al., 2001). The higher survival (75%) of pueruli at 19°C in the present study suggests that the required temperature changes in later stages of development, as is the case for late-stage *P. japonicus* (Matsuda and Yamakawa, 1997). It is important to note that replication was low in the present study and the effects of temperature on survival of final stage phyllosoma will require further research with larger numbers of animals

and at more temperatures in order to precisely model and predict the optimum temperature.

Although high survival was recorded for animals going through metamorphosis at 19°C, this was not the case during instar 17. Phyllosoma from the 19°C treatment had the lowest survival of 43% during instar 17, while 52% was the highest survival in the 23°C treatment. Observations suggested that mortality probably resulted from proliferation of *Vibrio* bacteria (vibriosis). Such mortality in *S. verreauxi* phyllosoma has previously been described by Diggles et al. (2000). Initial outbreaks of vibriosis typically follow an increase in water temperature and are more likely to occur at warmer water temperatures (>22°C) (Diggles et al., 2000). However, given that phyllosoma in the present study experienced a greater overall change in temperature (4°C) at a faster rate (0.3°C min^{-1}) when it decreased to 19°C compared to an increase to 25°C (2°C at a rate of 0.16°C min^{-1}), it is possible the larger change in temperature may have created a stronger physiological response in these phyllosoma and may explain the lower survival of phyllosoma in the 19°C treatment during instar 17. It may also be argued that, because of the higher mortality encountered in the 19°C treatment, the phyllosoma that attempted metamorphosis were stronger individuals than those in the other two treatments, which may have resulted in the higher survival of puerulus.

2.5.2 Routine metabolic rate

Animals usually display an acute response to a change in temperature that is characterised by $\dot{M}O_2$ exceeding initial rates (Newell and Branch, 1980). Following an acute response, metabolic rate stabilises and indicates acclimation has occurred. However, in many instances, partial acclimation occurs and the initial acclimated

metabolic rate fluctuates over time, before it approaches (but does not reach) the initial value obtained prior to acclimation (Precht, 1958).

Following transfer to a higher temperature, an organism's rate of reaction increases, whereas when an organism is exposed to a lower temperature, the rate of reaction decreases (Precht, 1958). The response to a change in temperature by *S. verreauxi* phyllosoma in the present study was characteristic of that observed in many crustaceans, with the R_r increasing at 25°C and decreasing at 19°C immediately after the change from the initial temperature of 23°C. Many marine organisms display an initial overshoot in $\dot{M}O_2$ as a stress response when they are exposed to a change in temperature (Bullock, 1955; Grainger, 1958; Prosser, 1973). Burton et al. (1981) suggested that comparing initial $\dot{M}O_2$ to final $\dot{M}O_2$ can indicate whether the organism has been stressed or damaged by the temperature change. If the $\dot{M}O_2$ rapidly returns to initial levels, this indicates the animal was not stressed and that this response represents the normal temperature compensation which occurs within the species thermal tolerance range (Precht, 1958). Therefore, the changes in R_r, either above (25°C) or below (19°C) pre-exposure levels after a change in temperature and the gradual return towards pre-exposure levels in the present study may be a typical physiological temperature compensation response, not a thermal stress response. As a result, all the temperatures investigated in the present study would be deemed to be within the thermal tolerance range of instar 17 *S. verreauxi* phyllosoma. Even though there is an immediate response to a change in temperature, the R_r after 30 h of exposure appears to be heading towards initial levels, representing the normal thermal acclimation processes of *S. verreauxi* phyllosoma.

Some variation in R_r was observed for phyllosoma in the 23°C treatment. Since these animals were not exposed to a temperature change and were held at 23°C

for the duration of the experiment, the R_r for this group should have been very similar over time. The variation in R_r measured for these phyllosoma may be explained by insufficient replication and the method of respirometry used. The method of static respirometry does not compensate for periods of activity, which may result in an overestimation of R_r. Perera et al. (2005) reported that there may be large variations in the routine metabolism of juvenile lobsters due to alternation of behaviour between inactive to short periods of fully active swimming. The primary reason phyllosoma may have increased their activity level in the present study is due to handling. Increased $\dot{M}O_2$ due to handling stress is a common occurrence in respirometry trials (Cech, 1990). The time required for metabolic rates to become stable following handling is referred to as the adaption phase (Herrmann and Enders, 2000) and respiratory trials should, therefore, exclude measurements during the adaption phase to prevent over-estimating metabolic rates (Herrmann and Enders, 2000; Steffensen, 2002). Even though phyllosoma were adapted to chambers overnight, an increase in $\dot{M}O_2$ may have occurred during sealing of the chambers. A method that eliminates problems associated with handling and periods of activity is intermittent flow-through respirometry, which permits repeated measurements of $\dot{M}O_2$ during short time intervals over extended periods (Steffensen, 1989). Intermittent flow-through respirometry also allows more accurate readings of metabolic rate, however, the technique needs refining before it is suitable for use with small larval crustaceans.

2.5.3 Feeding prior to metamorphosis

Daily feed intake of phyllosoma increases as a function of temperature (Tong et al., 2000), but plateaus at higher temperatures (Bermudes and Ritar, 2004). Overall feed intake (within an instar) may also decrease at higher temperatures due to accelerated development and shorter moult increments (Tong et al., 2000). Increased

metabolic rates and reduced feed intake at higher temperatures may result in an energy deficit, as elevated temperatures increase the amount of assimilated energy required for maintenance metabolism (Sweeney and Vannote, 1978). This increased energy requirement may explain why there was a significant difference in the number of days phyllosoma ceased feeding before metamorphosis in the present study. Phyllosoma in the 19°C treatment ceased feeding at least five days earlier than at 23°C and 25°C, suggesting that phyllosoma in the 23°C and 25°C treatments needed to continue feeding right up to metamorphosis to compensate for higher metabolic rates and the requirement to increase assimilation of energy. The increased energy requirements of phyllosoma in the 23°C and 25°C treatments may also lead to depletion of energy stores for metamorphosis and phyllosoma failing to reach the 'point of reserve saturation' (Anger and Dawirs, 1981). This is supported by Moss et al. (2001) indicating a decrease in temperature may lead to a decline in metabolic rate and allow phyllosoma to accumulate the required reserves for development to puerulus. Kittaka (2000) also observed that phyllosoma stop feeding a few days before metamorphosis when cultured at 20°C.

2.5.4 Cues for metamorphosis

Temperature change did not affect instar duration in the present study and indicated that temperature is not a cue for metamorphosis in *S. verreauxi*. This finding is consistent with Booth and Phillips (1994) who rejected the possibility that temperature could directly trigger metamorphosis due to the fluctuation experienced by phyllosoma in the wild during diurnal vertical migrations. There is sufficient evidence from laboratory culture and experiments on larval decapods and other taxa to suggest successful metamorphosis normally occurs after development through a certain number of instars and depends on the nutritional condition or level of energy

reserves of final stage phyllosoma (McConaugha, 1985; Kittaka et al., 1997; Jeffs et al., 2001). This is associated with the quantity and quality of food (McWilliam and Phillips, 1997). It is therefore likely that metamorphosis only occurs after the final stage phyllosoma has reached a specific level of stored energy reserves and has become competent (McWilliam and Phillips, 1997).

To my knowledge there has been no scientific evidence to support any previous theories of a cue for metamorphosis in spiny lobsters. In most marine crustacean larvae, the time of metamorphosis depends mainly on the duration of development from hatching to the period of metamorphic competence (Yoshimura, 2005). This is primarily controlled by genetic factors, but is also strongly influenced by extrinsic factors such as temperature and food. However, once larvae become competent they have the ability to extend their larval phase until they encounter a specific settlement cue or habitat (Rodriguez and Epifanio, 2000; Forward et al., 2001; Gebauer et al., 2003; Stanley et al., 2009). For example, the larvae of a number of crab species were found to orientate and swim toward ambient underwater sound originating from coastal reef habitats, which is likely to be an important settlement cue for the megalopae of these crab species (Stanley et al., 2009). But the term metamorphosis in lobster species refers to the physiological process of moulting from the final instar phyllosoma to the completely transparent puerulus stage, a development that is accompanied by drastic changes in morphology (Matsuda et al., 2003). Phyllosoma therefore develop through the zoeal phase and metamorphose to the larval decapodid puerulus phase (Anger, 2001). However, in other crustacean species such as crabs, metamorphosis refers to the transition from the final larval decapodid (megalopae) to the juvenile stage, which is associated with settlement (Anger, 2001; Stanley et al., 2009). As a result, research into time to metamorphosis

and metamorphosis cues for these crab species is concentrated around the settlement of the juvenile lifecycle phase. This raises the possibility that if a cue does exist for spiny lobsters, it may in fact be present in the transitional period between the puerulus and juvenile and not during metamorphosis from phyllosoma to puerulus, which was suggested by Goldstein and Butler (2009).

2.5.5 Conclusion

In the present study, whilst temperature was shown to affect the survival of phyllosoma at metamorphosis, a change in temperature was not a cue for metamorphosis. Given that 75% of the phyllosoma that metamorphosed at 19°C survived, in comparison to 33% at 23°C and 0% at 25°C, it is suggested that 23°C and 25°C are less effective temperatures for final stage *S. verreauxi* during metamorphosis. However, this was a preliminary study and further research is required to confirm the conclusions due to low replication and to determine the lower thermal tolerance limits of final stage phyllosoma and optimal culture temperature for maximum puerulus production. The increase (25°C) and decrease (19°C) in R_r compared to pre-exposure levels at 23°C appears to be a typical thermal acclimation response, indicating 19°C and 25°C are within the thermal tolerance range of instar 17 *S. verreauxi* phyllosoma.

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THE EFFECT OF STOCKING DENSITY ON GROWTH, METABOLISM AND AMMONIA-N EXCRETION OF THE SPINY LOBSTER *Sagmariasus verreauxi* FROM HATCH TO PUERULUS

Chapter III

3.1 Abstract

High stocking densities reduce the growth and survival of phyllosoma. By studying the metabolic physiology of phyllosoma in response to high stocking densities we can better understand the physiological mechanisms behind this reduced development and aid in the successful propagation of spiny lobsters. This study determined the effect of stocking density on the growth, development, routine metabolic rate (R_r), ammonia-N excretion rate, and energy storage and utilisation of the spiny lobster *Sagmariasus verreauxi* during ontogeny. Phyllosoma were cultured at two densities, with the High Density (HD) treatment (60 to 15 phyllosoma l^{-1}) being three times greater than the Low Density (LD) treatment (20 to 0.5 phyllosoma l^{-1}). Culture through the entire phyllosoma phase demonstrated that dry mass (DM) of phyllosoma was not affected by density until instar 17 when phyllosoma cultured at LD were significantly heavier (75.47 ± 4.18 mg) than at HD (63.69 ± 2.57 mg). Phyllosoma growth and development (total length, carapace width, and instar stage) was more advanced in LD phyllosoma after 108 d in culture. This advanced growth and development of LD phyllosoma was possibly due to increased interactions between phyllosoma and prey and reduction in feeding success of HD phyllosoma. Mass specific R_r was not affected by density and decreased through the early and mid-stages but it increased at instars 15 and 17. The increase in R_r in the final instars is likely due the physiological changes associated with metamorphosis and increased activity. Mass dependant R_r increased allometrically with body mass with a scaling exponent of 0.97 for LD phyllosoma and 0.90 for HD phyllosoma. Ammonia-N excretion rates increased significantly in instar 17 phyllosoma, representing a clear shift towards higher protein catabolism in the final instar phyllosoma. The shift in

energy substrate was also characterised by a decrease in the O:N ratio, which reflects the importance of final stage phyllosoma storing lipid as an energy reserve for metamorphosis and the non-feeding puerulus stage. These results revealed there is a shift in metabolism and energy storage and utilisation of instar 17 phyllosoma due to the substantial physiological changes and high energy requirements of phyllosoma in preparation for metamorphosis. Propagation of spiny lobsters should therefore aim to minimise energetic demands of late stage phyllosoma during this highly critical phase and provide optimum culture conditions. It is recommended that culture systems avoid high stocking densities to prevent reduced growth and development of phyllosoma, which decreases their ability to survive and successfully complete metamorphosis.

3.2 Introduction

There is great interest in phyllosoma culture due to the high commercial value and increasing demand of lobsters (Jeffs and Hooker, 2000). The evaluation of energy utilisation during larval development may indicate ontogenetic changes in growth efficiencies and reveal growth responses associated with specific culture conditions. Culture density is an important consideration in larval rearing of crustaceans because it may contribute to excessive expenditure of energy through increased physical interactions with prey and other phyllosoma and affect their ability to survive and successfully complete metamorphosis. Despite this, the effect of density on metabolism, energy storage and energy utilisation of phyllosoma has not been studied previously.

Oxygen consumption rates ($\dot{M}O_2$) represent overall aerobic metabolism, while ammonia-N excretion rates represent protein catabolism (Ikeda et al., 2000). The most common measure in respiratory studies is arguably the routine metabolic rate (R_r) (Cockcroft and Wooldridge, 1985), which ascertains how energy losses due to metabolism are influenced by environmental stressors (Lankin et al., 2008). Routine metabolic rate is defined as the intermediate state of metabolism measured in unfed animals displaying normal or spontaneous activity and can vary considerably due to unquantified activity levels (Anger, 2001). An increase in metabolic rate induced by stress uses energy within the metabolic scope that could otherwise be utilised for other physiological functions, such as growth and development (Fry, 1971; Brett and Groves, 1979; Priede, 1985). Stress associated with density may also affect O:N ratios as they are used to assess changes in energy substrate utilisation under various conditions (Corner and Cowey, 1968). According to the average elemental

composition of the main compound classes, an atomic O:N ratio with a minimum value of 7 are an indication that protein is being used an energy source (Ikeda, 1974). In contrast, if the substrate consists of equal amounts of protein and lipid this value will be about 24 (Ikeda, 1974), while higher values show an increasing utilisation of lipids and/or carbohydrates (Corner and Cowey, 1968). Consistently low O:N ratios have been observed in larval crustaceans (Chu and Ovsianico-Koulikowsky, 1994), indicating protein-based metabolism (Anger et al., 1989a), which has also been associated with stressful conditions (Pillai and Diwan, 2002). The O:N ratio has also been shown to decrease slightly during larval development of clawed lobsters (*Homarus americanus*), demonstrating an increasing importance in protein metabolism in later larval stages of this species (Anger et al., 1989a). This increased importance in protein metabolism may be due to preferential storage of lipids as an energy reserve for the energetically demanding processes of metamorphosis and settlement, which is similar to spiny lobsters storing lipid to fuel the non-feeding puerulus stage.

Body size is perhaps the most influential factor on the metabolic rate of an individual (Anger, 2001). The mass scaling exponent indicates the rate of change in metabolic rate with increasing body mass and ranges between 0.67 and 1.0, which indicates that metabolic rate is relative to the change in animals' body surface area (0.67), to the animals' volume (1.0), or somewhere in between (Schmidt-Nielsen, 1984). Ontogenetic variation in the mass scaling exponent may be caused by changes in the body shape and volume relationship; the proportions of living protoplasm and biomass of metabolically inert biochemical components; certain extrinsic factors; and physiological stress (Anger, 2001). In several studies with larval crustaceans, exponent values above 1.0 were usually associated with physiological stress. For

example, high exponent values were observed in caridean shrimp larvae exposed to unfavourable temperatures and salinities (Yagi et al., 1990), and exponents above 1.0 also occurred in brachyuran crab larvae cultured at extreme temperatures (Dawirs, 1983).

Changes in R_r and ammonia-N excretion rates during the entire course of phyllosoma development have rarely been measured. The aim of the present study was to investigate the stage-specific changes in metabolism, with regard to R_r and ammonia-N excretion rates during ontogeny of *Sagmariasus verreauxi* in order to evaluate the energetic demands through larval development. Also, the effect of culture density on metabolism, energy storage and energy utilisation, with regard to distribution to metabolism or retention, was investigated.

3.3 Materials and methods

3.3.1 Experimental animals

Broodstock were fed a combination of fresh whole blue mussels (*Mytilus edulis*) and commercial prawn pellet (Higashimaru, Vital No. 12, http://www.k-higashimaru.co.jp/) twice a week. Broodstock were held in captivity in a 4,000 l fibreglass tank year round under a regime of ambient photoperiod and water temperature (11°C-19°C), 33-35 psu salinity, pH approximately 8.1, and 90-100% oxygen saturation at the Institute for Marine and Antarctic Studies (IMAS), Taroona, Hobart. Phyllosoma used in this experiment were hatched on the 8th of February 2009 from one female weighing approximately 2.0 kg.

3.3.2 Larval culture

Triplicate 7 ml sub-samples of newly-hatched phyllosoma were counted from a 20 l bucket to estimate density and then stocked into 50 l cylindrical tanks with flow-through seawater filtered to 1 µm and treated with ozone and ultra-violet irradiation according to Jensen et al. (2011) and maintained at 21-23°C using an industrial heat/chill unit (Carrier, C010PHH7AA, Australia). Four replicate tanks were initially stocked at Low Density (LD) (20 phyllosoma l^{-1}) and High Density (HD) (60 phyllosoma l^{-1}). However, due to high mortalities in the LD treatment caused by an acute ozonation problem during instar 9, this treatment was reduced to three replicates in order to maintain densities between treatments. Phyllosoma were cultured through successive larval stages to the final instar (instar 17), and the densities progressively reduced, but the density of the HD treatment was three times greater than the LD treatment throughout the experiment. Stocking densities of the

LD treatment were reduced to 10, 5, 2.5, 1.25, and 0.5 l^{-1} at instars 3, 6, 9, 12, and 15, respectively, by randomly culling phyllosoma. Stocking densities of the HD treatment were reduced to 30, 15, 7.5, 3.75, and 1.5 l^{-1} at equivalent instars.

Phyllosoma were fed a combination of freshly prepared blue mussel (*Mytilus edulis*) gonad (~ 5 mm diameter) once daily and on-grown *Artemia* of 5.0 - 8.0 mm in length three times a week. Despite extensive water treatment, the daily addition of food increased bacterial levels and tanks (50 l) were cleaned every 14 days to reduce accumulation of bacteria and fouling.

3.3.3 Feed production

Artemia nauplii were stocked in 670 l tanks at 5 *Artemia* ml^{-1} and on-grown to approximately 8.0 mm in flow-through tanks receiving a diet of blended brine shrimp food (consisting of rice pollard, soyflour and wheat flour; Eyre Peninsula Aquafeeds Pty Ltd, South Australia) and algae (T. *Isochrysis* and *Chaetoceros muelleri*). Prior to feeding to phyllosoma, gut bacteria in *Artemia* were minimised by purging in dense cultures of *C. muelleri* (10^7 cells ml^{-1}), as described by Tolomei et al. (2004), and 400 ppm formalin for 30 min before rinsing with filtered seawater and feeding to phyllosoma culture tanks. *Artemia* feeding rates were based on phyllosoma density and kept consistent between treatments with the HD treatment receiving three times more (1.38 g dry mass, DM) than the LD treatment (0.46 g DM) to minimise the density effects of starvation (Minagawa and Murano, 1993; Mikami, 1995). The length:mass relationship of *Artemia* cultured at IMAS was used to determine DM of ration based on *Artemia* length. Uneaten *Artemia* remaining from the previous meal were flushed from the tanks prior to feeding the next meal of *Artemia*.

Meals of fresh pieces (5.0 mm in diameter) of blue mussel (*M. edulis*) were kept consistent between treatments (0.25 ml $phyllosoma^{-1}$) with the HD treatment

receiving three times more mussel than the LD treatment. Uneaten mussel was siphoned from culture vessels each day before feeding the next meal of mussel.

3.3.4 Respirometry

Routine metabolic rate of intermoult phyllosoma from both HD and LD treatments were determined at instars 1, 3, 6, 9, 12, 15, and 17. A sample of phyllosoma (n= 20) from each tank was measured at regular intervals for total length (TL) and carapace width (CW) using a profile projector (Nikon 6C, Japan) and staged according to Kittaka et al. (1997) under a dissecting microscope (AIS Optical) at 10-40x magnification to determine intermoult periods. Phyllosoma were individually stocked into 1.6 l cylindrical tanks and then observed for moulting events to ensure a direct comparison of the same instar was achieved for all samples. They were then monitored before being placed into respirometers to ensure they were in the intermoult phase, which was defined as the halfway stage of a given instar (in days) based on previous laboratory observations by Kittaka et al. (1997). Phyllosoma were starved for 24 h prior to being placed in respirometers to clear the digestive tract of food and faeces and eliminate variability of measurements associated with specific dynamic action (SDA). To avoid interference from possible circadian behavioural patterns, $\dot{M}O_2$ of phyllosoma was always measured during daylight hours between 0800 h and 1800 h. Throughout the respirometry trial, the oxygen saturation was kept above 70% (Ikeda et al., 2000).

Phyllosoma $\dot{M}O_2$ was measured in an automated intermittent flow-through respirometer system which consisted of either 6 ml or 39 ml respirometer chambers. Smaller 6 ml respirometry chambers were used for $\dot{M}O_2$ measurements from instar 1 to 12 and the 39 ml chambers were used for instars 15 and 17. Respirometers were stocked with 20, 15, 15, 5, and 1 phyllosoma at instars 1, 3, 6, 9, and 12-17,

respectively. A total of four replicate respiratory measurements were taken for instars 1 to 9 and eleven replicates were taken from instars 12 to 17 at each density. The chambers were immersed in a 20 l ambient tank (temperature bath) to ensure the temperature remained constant for each measurement. The seawater in the 20 l tank was kept air-saturated with a flow-through rate of 3 exchanges h^{-1}. Temperature was regulated in the ambient tank using a 150 watt aquarium heater (Aquarium Regler-Heizer, N2350). The oxygen content in the chamber was measured with a luminescent dissolved oxygen optode (Hach LDO, HQ40d, Hach Company, USA) that logged dissolved oxygen recorded every 60 s. A recirculating peristaltic pump (Harvard Apparatus MPII, SDR Clinical Technology, NSW, Australia) ensured proper mixing of seawater inside the respirometer and adequate flow passed the oxygen probe. A three-way solenoid valve (Burkert, Germany) exchanged the water inside the respirometer with water from the ambient tank every 10 min allowing a $\dot{M}O_2$ measurement every 20 min. An identical blank control chamber without a phyllosoma was used to simultaneously determine background $\dot{M}O_2$. Oxygen consumption rate was expressed in mg O_2 g DM^{-1} h^{-1} after the subtraction of background $\dot{M}O_2$ obtained from the control respirometer. Oxygen consumption rate was measured over a period of 2 h for instars 1 and 3, 4 h for instars 6 and 9 and 8 h from instars 12 to 17 equating to between 6 and 24 $\dot{M}O_2$ measurements for each specimen. Activity of phyllosoma was restricted by the confines of the respiratory chamber. Routine metabolic rate was defined as the average of all $\dot{M}O_2$ readings for each phyllosoma (Herrmann and Enders, 2000).

Following measurements of $\dot{M}O_2$ the seawater in each chamber was sampled for ammonia-N analysis (see below) and the TL, CW, instar stage, and DM of each phyllosoma were measured. Dry mass was determined after rinsing phyllosoma in

0.5M ammonium formate to remove any salt and drying in an oven for 24 h at 60°C. The mass of each sample was measured to the nearest 10 μg on a precision balance (Mettler AT261 DeltaRange, Metler-Toledo, Switzerland).

3.3.5 Ammonia-N excretion rate

At the end of each respiratory measurement seawater samples were incubated for 20 min in the 6ml chambers and 30 min in the 39 ml chambers, during which time $\dot{M}O_2$ was simultaneously recorded and used to determine the O:N ratio. The total chamber seawater volume was then drawn from each chamber, sealed in 10% HCL acid-washed glass vials with a drop of 10% chloroform added to prevent bacterial accumulation and stored at -4°C prior to analysis. The ammonia-N concentration in these samples was determined using a salicylate-hypochlorite method following the procedure of Bower and Holm-Hansen (1980) and expressed in mg NH_4-N g DM^{-1} h^{-1} after the subtraction of ammonia-N concentration in control vials.

Atomic O:N ratios were determined by the division of oxygen consumption (mg O_2 g DM^{-1} h^{-1}) and ammonia-N excretion rates (mg NH_4-N g DM^{-1} h^{-1}), using the atomic masses of O_2 (32.00) and N (14.01).

3.3.6 Data analysis

Oxygen consumption rates of phyllosoma and controls were determined using linear regression of the rate of decline in dissolved oxygen concentration for the final 8 min of every 10 min closed respirometer cycle. Only measurements with regression coefficients (R^2) above 0.95 were used to calculate $\dot{M}O_2$ of phyllosoma and background $\dot{M}O_2$. Residual plots were used to explore normality and homogeneity of data. Outliers were identified using boxplots and removed from statistical analysis. Differences of mean R_r, ammonia-N excretion rates, and O:N ratio between densities

and instar stages were compared using two-way analysis of variance (ANOVA) and significant differences identified using Tukey's HSD tests for post-hoc multiple comparisons. Where no interaction between two factors occurred, differences between densities or instars were reported. Where significant differences occurred one-way ANOVA was used to test for differences between instars for each density treatment. Allometric relationships were compared using linear regression analysis with significant differences in line slopes and y-intercepts determined using StatistiXL version 1.8 (Copyright Microsoft Excel 2007). Growth measurements were compared between densities using an independent sample t-test and where no significant differences occurred two-way ANOVA is presented. Exponential regressions were used to describe the relationship between growth parameters and instar stage. Linear and power regressions were used to analyse the relationship between growth parameters and culture day using SigmaPlot version 12 (2011 Systat Software Inc.). ANOVA and t-tests were performed using SPSS version 16.0 (2007 SPSS Inc.). The level of significance for all analyses was determined at $P<0.05$. Data are presented as mean ± standard error (SE) unless stated otherwise.

3.4 Results

3.4.1 Growth

The individual DM, TL, and CW of phyllosoma increased exponentially with development for both LD and HD treatments (Table 3.1, Fig. 3.1). There was no significant difference in DM of phyllosoma up to instar 15 (two-way ANOVA; F= 0.497, df 1, 99, *P*= 0.483). However, DM of instar 17 phyllosoma in the LD treatment were significantly heavier than those in the HD treatment (t= -2.400, df 22, *P*= 0.025). Total length (two-way ANOVA; F= 0.768, df 1, 99, *P*= 0.383) and CW (two-way ANOVA; F= 0.628, df 1, 99, *P*= 0.430) compared at instar development stage were not significantly affected by density. However, density affected the rate of growth and development of phyllosoma, with TL (two-way ANOVA; F= 87.473, df 1, 61, *P*< 0.001, Fig. 3.2A), CW (two-way ANOVA; F= 90.793, df 1, 61, *P*< 0.001, Fig. 3.2B) and instar stage (two-way ANOVA; F= 72.26, df 1, 61, *P*< 0.001, Fig. 3.2C) after 108 d in culture being significantly greater in the LD treatment. The TL and CW increased linearly and instar stage increased as a power relationship with culture day for both LD and HD treatments (Table 3.1).

3.4.2 Routine metabolic rate

There was no significant interaction between density and instar stage for mass specific R_r of phyllosoma (two-way ANOVA; F= 0.799, df 6, 56, *P*= 0.576). Mass specific R_r of phyllosoma was also not significantly affected by density (two-way ANOVA; F= 1.269, df 1, 56, *P*= 0.266) and at both densities decreased rapidly between instar 1 and 3 before stabilising and increasing in the later larval stages (Fig. 3.3A). However, instar stage had a significant impact on mass specific R_r of

phyllosoma (two-way ANOVA; F= 41.247, df 6, 56, $P< 0.001$). The R_r of instar 1 phyllosoma was significantly higher than all other instars and R_r of instar 17 phyllosoma was significantly higher than instar 6 and 12 phyllosoma.

There was a positive allometric relationship between mass independent R_r and body mass of phyllosoma for both LD (F= 832.349, df 1, 6, $P< 0.001$) and HD (F= 5997.100, df 1, 6, $P< 0.001$) phyllosoma (Fig. 3.4). Body mass explained 97.5% of the variation in R_r of phyllosoma in the LD treatment, and 97.3% of the variation in the HD treatment. The mass scaling exponent for LD phyllosoma (0.97) was higher than for the HD (0.90) but there were no significant differences in the line slopes (F= 0.561, df 1, 30, $P= 0.460$) and intercept with the y-axis (F= 0.021, df 1, 31, $P= 0.885$) between densities.

In order to compare species-specific differences in R_r, data from mixed stages of crustacean larvae and several spiny lobster species, including *S. verreauxi* cultured at LD from the present study, were plotted against DM (Fig. 3.5). A regression line (referred to as the "crustacean" line hereafter) was calculated for mixed stages of crustacean larvae and compared to a regression line (referred to as the "lobster" line hereafter) and its 95% confidence interval (CI) for instar 1 to 17 *S. verreauxi* combined with mixed stages of several other spiny lobster species. There was a positive allometric relationship between mass independent R_r and body mass (DM) for both the crustacean line (F= 2066.073, df 1, 150, $P< 0.001$) and lobster line (F= 1748.271, df 1, 21, $P< 0.001$). Body mass explained 86% of the variation in R_r of crustacean larvae and 95% of the variation in spiny lobster species. The regression slopes were significantly different between the crustacean line and lobster line (F= 1047.308, df 1, 169, $P< 0.001$), with the R_r of spiny lobster species significantly lower than for crustacean larvae.

3.4.3 Ammonia-N excretion rate

There was a significant interaction between density and instar stage for ammonia-N excretion rates of phyllosoma (two-way ANOVA; F= 4.156, df 6, 98, *P*< 0.001). The ammonia-N excretion rate of phyllosoma in the LD treatment was significantly affected by instar stage (one-way ANOVA; F= 6.957, df 6, 48, *P*< 0.001) but not in the HD treatment (one-way ANOVA; F= 2.016, df 6, 48, *P*= 0.085). The ammonia-N excretion rates of instar 17 phyllosoma were significantly higher than for instar 9, 12 and 15 phyllosoma. The ammonia-N excretion rate of phyllosoma was not significantly between densities (two-way ANOVA; F= 0.105, df 1, 98, *P*= 0.747) and remained relatively constant through larval development, but increased steeply from instar 15 to instar 17 (Fig. 3.3B).

3.4.4 O:N ratio

There was no significant interaction between density and instar stage for the O:N ratio of phyllosoma (two-way ANOVA; F= 1.034, df 6, 96, *P*= 0.409, Fig. 3.3C). The O:N ratio was also not significantly affected by density (two-way ANOVA; F= 0.254, df 1, 96, *P*= 0.616, Fig. 3.3C). However, the O:N ratio was significantly different between phyllosoma instars (two-way ANOVA; F= 8.797, df 6, 96, *P*< 0.001). The O:N ratio of instar 15 phyllosoma was significantly higher than instar 3, 6, 12, and 17 phyllosoma.

Table 3.1. Details of exponential ($y= ae^{bx}$) regressions describing the relationship between instar development stage (Instar) and dry mass (DM), total length (TL), and carapace width (CW); linear ($y= ax + b$) regressions describing the relationship between age (Day) and total length (TL), and carapace width (CW); and power ($y= a*x^{b}$) regressions describing the relationship between age (Day) and mean instar stage of *Sagmariasus verreauxi* phyllosoma cultured at Low Density (LD) or High Density (HD) presented in Figs. 3.1 and 3.2.

		Density	a	b	R^2	df	F	*P*
Instar	Dry mass (mg)	Low	0.273	0.331	0.99	6	15603.74	<0.001*
		High	0.624	0.274	0.99	6	553.97	<0.001*
	Total length (mm)	Low	2.451	0.158	0.99	6	3601.92	<0.001*
		High	2.602	0.152	0.99	6	1753.06	<0.001*
	Carapace width (mm)	Low	1.613	0.148	0.99	6	1540.45	<0.001*
		High	1.660	0.145	0.99	6	772.29	<0.001*
Day	Total length (mm)	Low	0.136	0.608	0.98	7	266.18	<0.001*
		High	0.113	1.476	0.99	7	626.73	<0.001*
	Carapace width (mm)	Low	0.079	0.472	0.98	7	351.32	<0.001*
		High	0.066	0.946	0.99	7	563.70	<0.001*
	Mean instar	Low	0.430	0.691	0.99	7	1099.48	<0.001*
		High	0.577	0.618	0.99	7	533.41	<0.001*

*indicates significant regression ($P<0.05$).

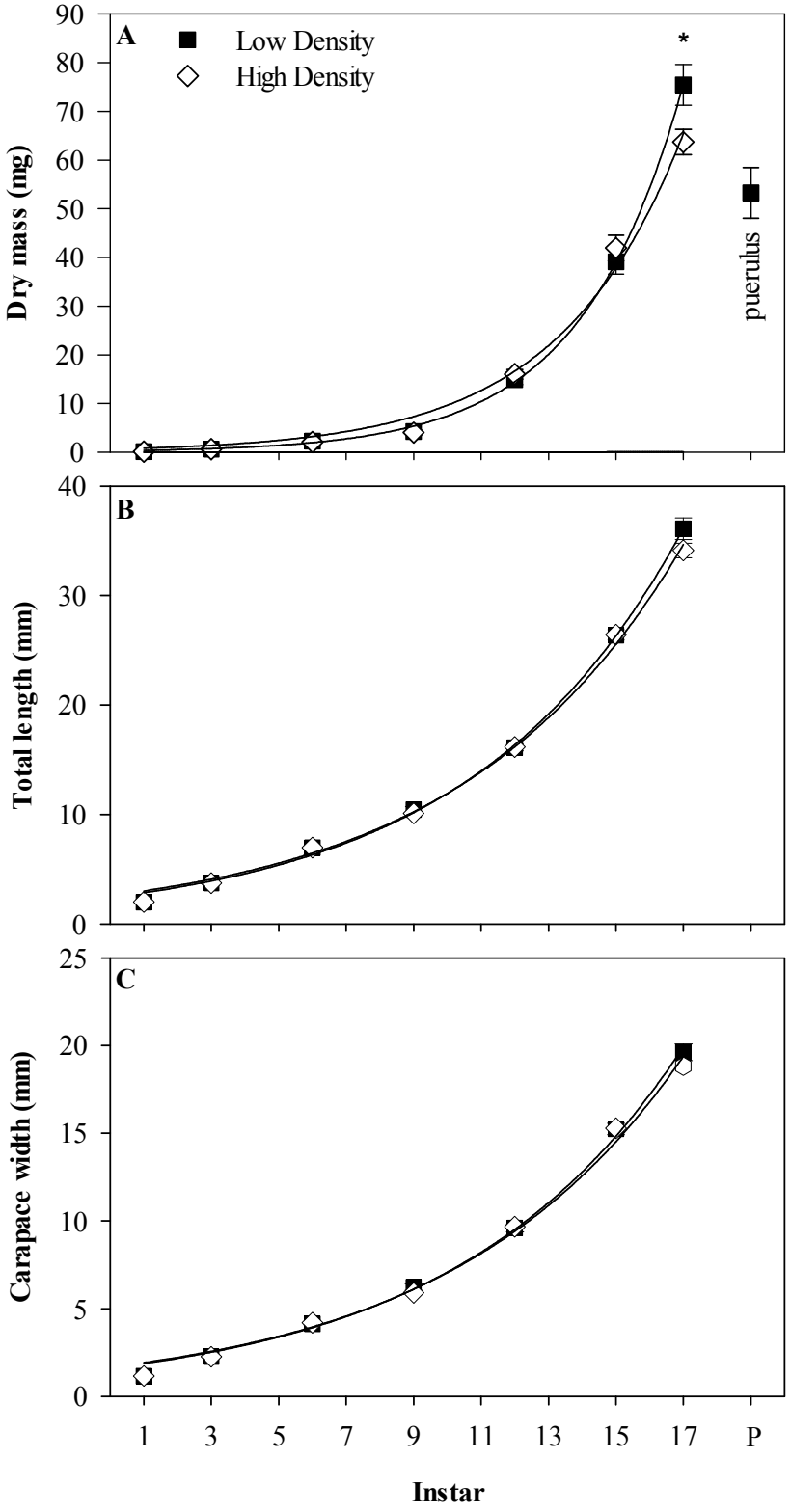

Figure 3.1. Relationship between: (A) dry mass (DM); (B) total length (TL); (C) carapace width (CW) and instar development stage of *Sagmariasus verreauxi* phyllosoma cultured at Low Density (LD) or High Density (HD). P denotes puerulus stage. Plots bearing asterisks are significantly different between densities at the instar indicated (P<0.05). Values are mean (±SE). Details of regressions are given in Table 3.1.

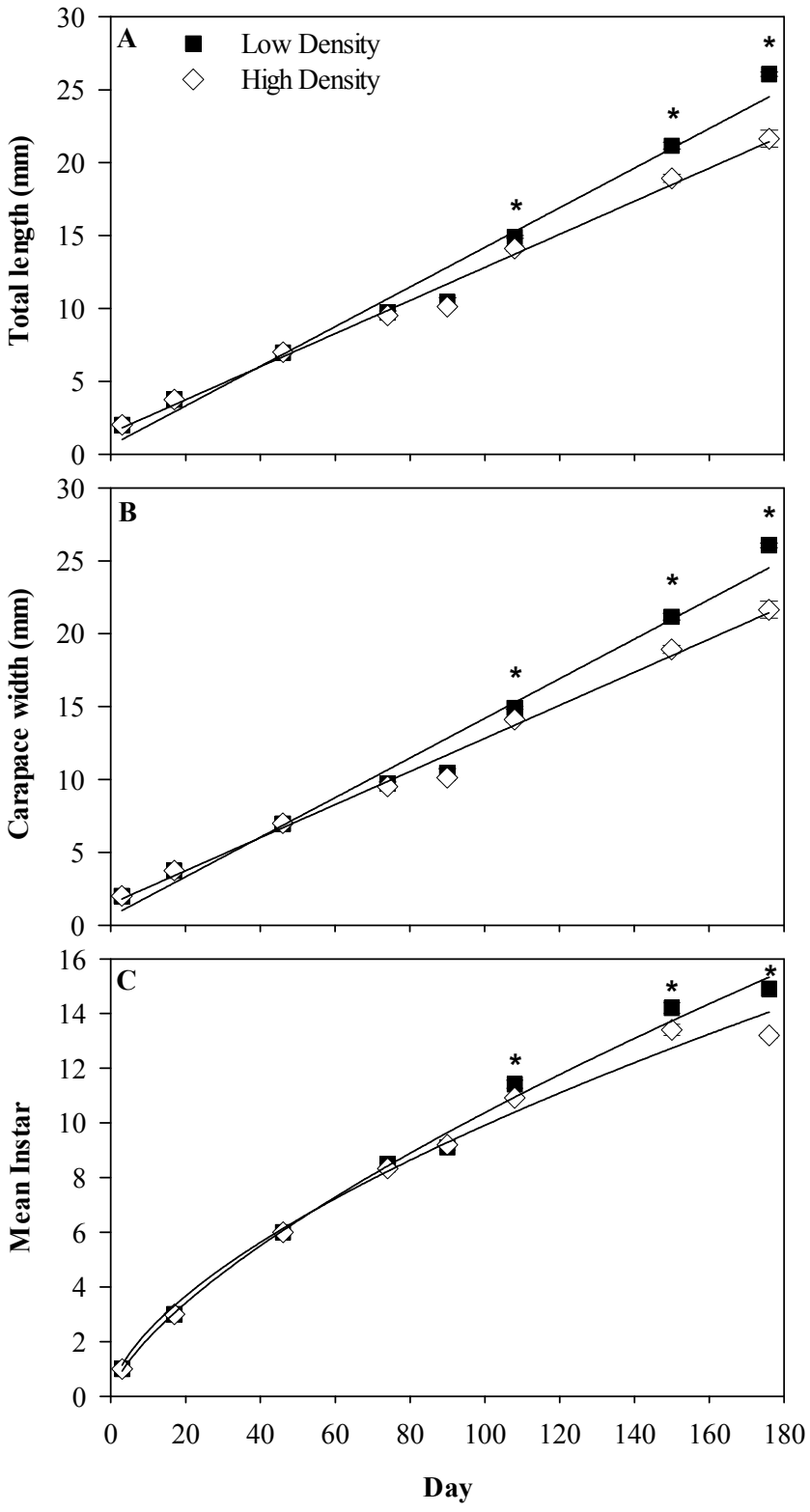

Figure 3.2. (A) Total length (TL); (B) carapace width (CW) and; (C) mean instar stage of *Sagmariasus verreauxi* phyllosoma at different days from hatch when cultured at Low Density (LD) or High Density (HD). Plots bearing asterisks are significantly different between densities (P<0.05). Values are mean (±SE). Details of regressions are given in Table 3.1.

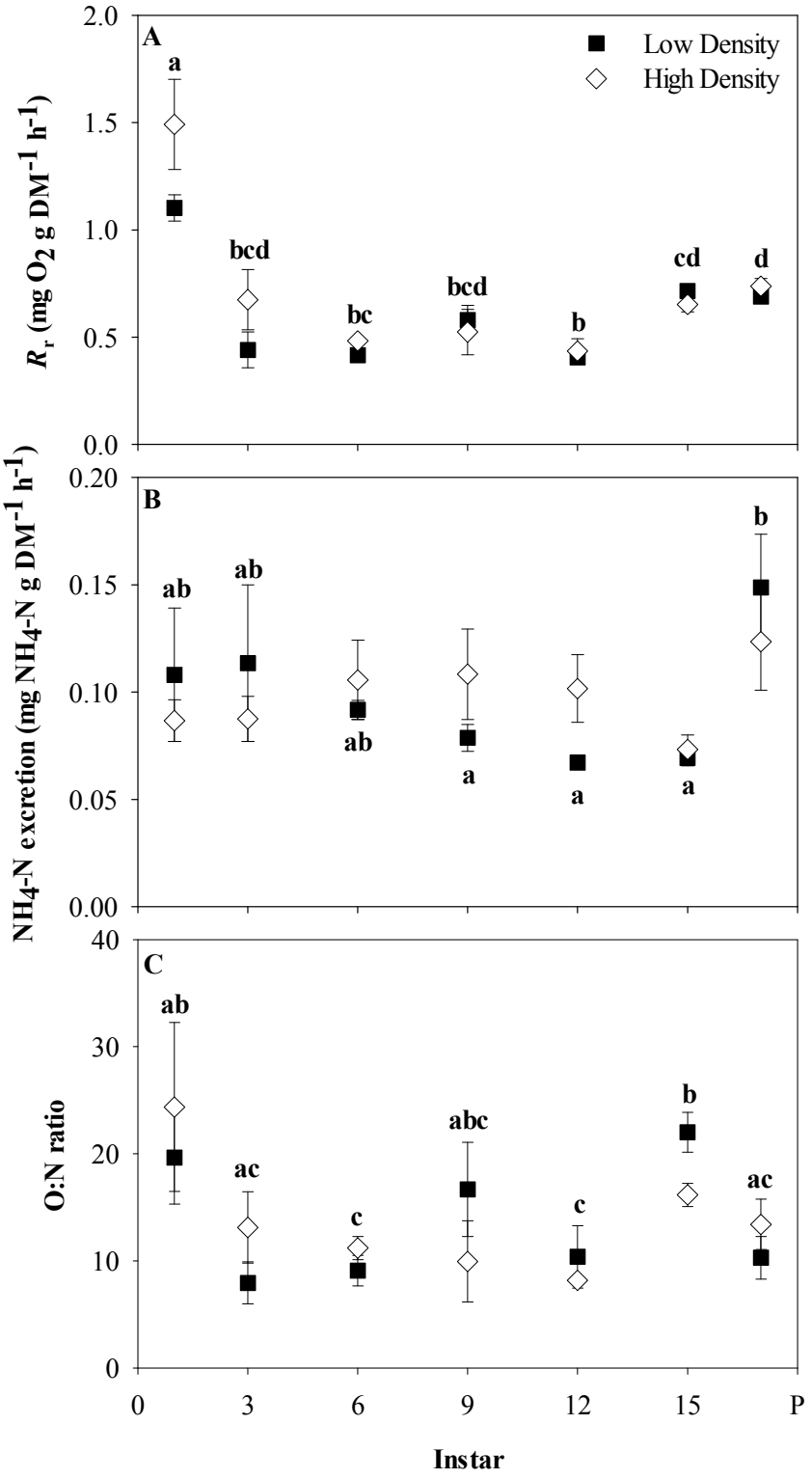

Figure 3.3. Developmental changes in: (A) routine metabolic rate (R_r); (B) ammonia-N excretion (NH_4-N) rate and; (C) atomic O:N ratio of *Sagmariasus verreauxi* phyllosoma cultured at Low Density (LD) and High Density (HD). P denotes puerulus stage. Data points bearing different superscripts are significantly different (P<0.05). Values are mean (±SE).

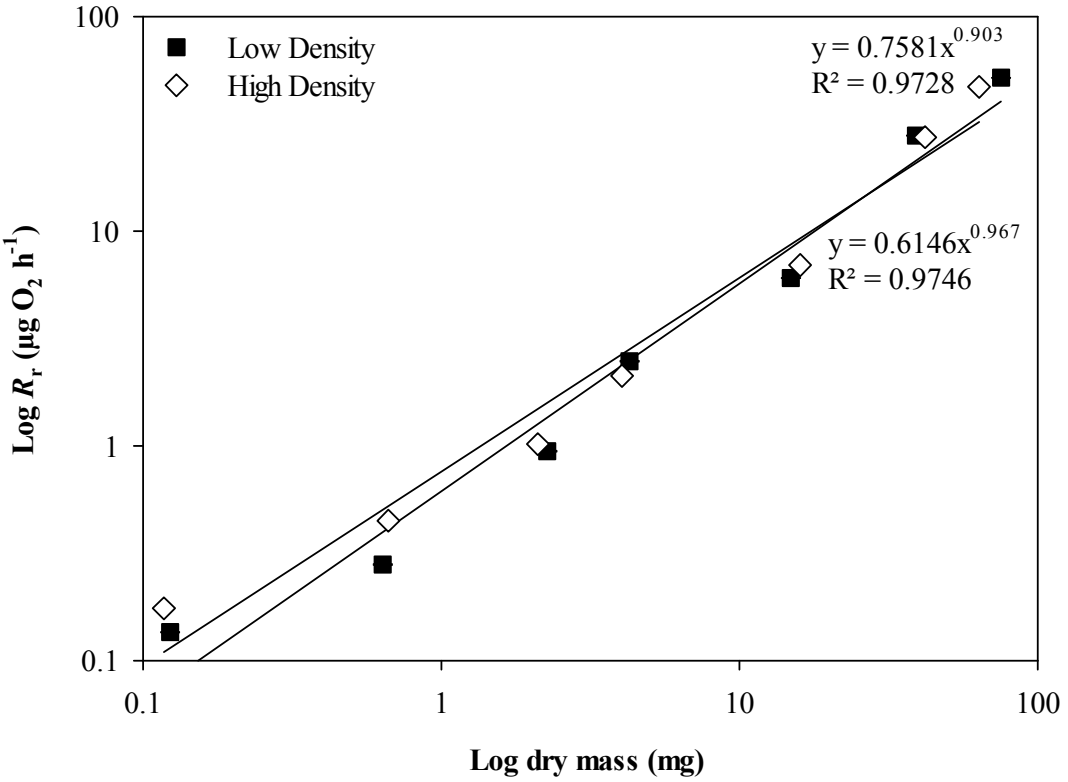

Figure 3.4. Allometric relationship between log of dry mass (DM) (mg) and routine metabolic rate (R_r) (µg O_2 h^{-1}) of *Sagmariasus verreauxi* phyllosoma cultured at Low Density (LD) and High Density (HD). Values are mean (±SE).

3.5 Discussion

3.5.1 Routine metabolic rate

Routine metabolic rate of *S. verreauxi* phyllosoma was significantly higher for newly-hatched phyllosoma compared to all other phyllosoma stages and was above the allometric trend line for the relationship between R_r and body mass (Fig. 3.4). High R_r of newly-hatched larvae have previously been recorded in different penaeid larvae (Kulkarni and Joshi, 1980; Chu and Ovsianico-Koulikowsky, 1994; Lemos and Phan, 2001) and crab larvae (Jacobi and Anger, 1985). The high mass specific R_r of instar 1 phyllosoma could be attributed to a decrease in the surface area: volume ratio of phyllosoma which limits gas exchange and digestive ability (Childress and Somero, 1990), or the transitions in lifestyle, particularly activity, throughout planktonic development (Lemos and Phan, 2001). Instar 1 phyllosoma are strongly photopositive, rising to the surface shortly after hatching to exploit surface currents for offshore transport (Ritz, 1972). Because measurements of R_r were carried out during daylight hours, this may have resulted in higher activity of phyllosoma following hatch-out. The high R_r of instar 1 phyllosoma may also be attributed to a reduced buoyancy of larvae associated with body composition (Jeffs et al., 1999) and reduced size of appendages, causing an increase in swimming activity. These changes in morphology and metabolic activity result in a reduction in oxygen demand throughout development (Capuzzo and Lancaster, 1979a; Chu and Ovsianico-Koulikowsky, 1994).

The observed increase in R_r during later phyllosoma stages opposes the expected decrease in mass-specific R_r for phyllosoma within this mass range and may be unique to *S. verreauxi* phyllosoma. Anger et al. (1989b) observed a decrease in

metabolic rate of *Hyas araneus* prior to the megalopae stage, which like puerulus displays negative growth, and suggested that this decrease may be a general occurrence among brachyuran larvae (Anger and Jacobi, 1985; Jacobi and Anger, 1985). In the present study, the gradual increase in R_r at instar 15 and 17 coincides with the development of gills and pleopods (Kittaka et al., 1997), which may facilitate greater gas exchange (Spicer, 1995). In contrast, Lemos and Phan (2001) reported that the metabolic rate of penaeid shrimp larvae decreased with the development of gills, which was attributed to enhanced gas exchange efficiency and decrease in swimming activity. Since the larval development of spiny lobsters deviates from other decapods (Williamson, 1988), it is more likely that increased metabolic rates in later larval stages, especially in instar 17 phyllosoma, are due to the substantial physiological changes and high energy requirements and oxygen demand in preparation for the morphological changes associated with metamorphosis (Lemmens, 1994a; Jeffs et al., 2001). The transition from the zoeal (early larval stages) to the decapodid (late larval stage) stage in many penaeid and crab species is not truly metamorphic (Anger, 2001). In most penaeid species, juvenile characters are attained gradually over a variable number of moults and many crab species pass through two metamorphoses (Anger, 2001). Matsuda and Takenouchi (2006) also found that the body length and growth index increased more rapidly in late stages of the spiny lobster *Panulirus japonicus* in relation to early and mid-stages. Alternatively, the elevated R_r may be due to increased swimming activity as several papers have suggested that late stage phyllosoma have the ability to swim towards shore (Chiswell and Booth, 1999; 2005; 2008). The pleopods in late stage phyllosoma are nearly as well developed as in pueruli, which could be used in conjunction with exopodal setai beating to allow them to swim (Chiswell and Booth, 1999).

3.5.2 Growth

The physiological responses of marine species to stocking density can vary considerably (Smith and Ritar, 2006). In predatory fish species, aggressive territorial behaviour can arise even at low stocking densities, which results in reduced feeding and growth (Kestemont et al., 2003). However, this type of behaviour has not previously been reported in larval crustaceans (Smith and Ritar, 2006). *S. verreauxi* phyllosoma cultured at LD accumulated more DM and were longer and wider than phyllosoma at HD. Phyllosoma in the LD treatment also had shorter instar durations and were consequently more advanced in regard to instar stage compared to the HD treatment. Instar duration in larval decapod crustaceans is often considered to be a sensitive indicator of unfavourable conditions (Dawirs, 1983). The longer instar durations of HD phyllosoma may suggest the culture conditions experienced in the HD treatment were unfavourable for growth and development of phyllosoma. Anger (2001) proposed larvae which have been cultured under optimal conditions should develop through the shortest possible progression of stages, whereas weak or stressed larvae will develop through a higher number of instars and may show morphological deformity. This is also consistent with Emmerson and Andrews (1981), who found development time increased as a function of stocking density in *Penaeus indicus* and growth and survival decreased with increasing density.

The reduced growth of phyllosoma in the HD treatment was not a result of limited food availability because phyllosoma were fed relative amounts between the LD and HD treatments. However, reduced growth and development of phyllosoma in the HD treatment may have resulted from reduced feeding durations and feed intake caused by increased interaction between phyllosoma. This is consistent with Smith and Ritar (2006) who found that growth and survival of *Jasus edwardsii* phyllosoma

was significantly reduced at densities above 40 l^{-1}. Smith and Ritar (2006) also noted that excessive clumping in high density, low flow cultures, could disrupt feeding behaviour of phyllosoma and impair their ability to obtain sufficient nutrition to compensate for reduced feed intake. This reduced feeding success is due to phyllosoma in high density cultures becoming entangled, causing physical damage to limbs and influencing their ability to actively capture prey. Phyllosoma that sustain physical damage during moulting and prior to their exoskeleton hardening may also acquire moult deformities, which results in prolonged instar durations and usually death at the following moult (Smith and Ritar, 2006).

3.5.3 Ammonia-N excretion rate

Ammonia-N excretion rates have rarely been measured during the entire larval phase of any spiny lobster species. Ammonia-N excretion rates remained reasonably constant throughout larval development of *S. verreauxi*, apart from a considerable increase at instar 17. This is similar to the near constant ammonia excretion observed during development from zoea I to megalopa in *Carcinus maenas* (Harms et al., 1994). However, high excretion rates have been recorded in early larval stages of *Farfantepenaeus paulensis*, reaching a maximum of 1.026 mg NH_4-N g^{-1} DM h^{-1} at protozoea II (Lemos and Phan, 2001). A significant ontogenetic decline in ammonia-N excretion rates was also reported during early instars of *J. edwardsii* (Bermudes and Ritar, 2004; Bermudes et al., 2008) and in *Macrobrachium rosenbergii* (0.00167 to 0.00007 mg NH_4-N g^{-1} DM h^{-1}) (Agard, 1999). Conversely, Ikeda et al (2011) observed low (0.76 mg NH_4-N $individual^{-1}$ h^{-1}) ammonia-N excretion rates of instar 1 *Panulirus ornatus*, which may have been due to allocation of protein to somatic growth rather than to metabolism. The significant variability in ammonia-N excretion rates of crustaceans not only compared to the present study but also between other

studies, may be attributed to measurement techniques. It is also likely that the ontogenetic patterns observed in ammonia-N excretion rates between spiny lobster species reflect species-specific differences in nutritional functions (Ikeda et al., 2011), because an increase in ammonia-N excretion during the final instar appears to be unique to *S. verreauxi* phyllosoma.

3.5.4 O:N ratio

The O:N ratios measured for different phyllosoma instars in the present study ranged from 7.9 to 22.0 in the LD treatment, and 8.2 to 24.4 in the HD treatment, suggesting that protein catabolism is the principal source of energy for larval stages fed *Artemia* and mussel gonad, but lipid sources may also be utilised to some extent. These results contrast those of Ikeda et al. (2011) who suggested metabolism during ontogeny of *P. ornatus* was lipid/carbohydrate-orientated. This is potentially due to the faster growth rate of *P. ornatus* (cultured at higher temperatures and with fewer instar stages), which may lead to a rapid turnover in protein reserves allocated to construction and repair of tissues associated with frequent moulting.

The variation in O:N throughout development of *S. verreauxi* phyllosoma is a consequence of both variations in $\dot{M}O_2$ and ammonia-N excretion rate, and may be associated with developmental changes in behaviour and body composition. Since ammonia-N excretion rates remained reasonably constant during larval development (until instar 17), variations in the O:N ratio are more strongly reflected by changes in $\dot{M}O_2$. In early instar 1 phyllosoma, the majority of energy can potentially be obtained from the catabolism of lipoprotein yolk reserves (Richard et al., 1979; Agard, 1999) characterised by high O:N ratios, although phyllosoma begin feeding immediately after hatching and may have little dependence on yolk reserves (Johnston and Ritar, 2001). However, because phyllosoma were starved prior to estimating R_r in the

present study to eliminate the effects of SDA, instar 1 phyllosoma may have had a higher dependence on yolk reserves compared to previous studies. Lower O:N in subsequent stages may correspond to a shift to protein catabolism after the exhaustion of these lipid reserves (Anger 1986; Harms and Anger, 1990; Harms et al., 1991; Chu and Ovsianico-Koulikowsky, 1994; Lemos and Rodriguez, 1998). Alternatively, although the diet was kept constant during larval development of *S. verreauxi*, some variation in the O:N ratio may have occurred due to phyllosoma assimilating different proportions of mussel gonad and *Artemia* at different larval stages (Matsuda et al., 2009). Despite this, the initial high O:N ratio of *S. verreauxi* phyllosoma (19.7 at LD and 24.4 at HD), followed by a reduction during subsequent stages of development, is consistent with trends found in *H. araneus* (Anger et al., 1989a) and *H. americanus* (Capuzzo and Lancaster, 1979a; Sasaki et al., 1986). However, the increased catabolism of protein during the final instar of *S. verreauxi*, which was characterised by a decrease in the O:N ratio between instar 15 and 17, does not commonly occur in other crustaceans. This increased catabolism of protein by instar 17 phyllosoma is possibly due to the importance of storing lipid for use as an energy reserve during the non-feeding puerulus stage (Jeffs et al., 1999; Jeffs et al., 2001). Phyllosoma preferentially store lipids for endergonic processes (Johnston et al., 2004), which may be an essential physiological survival strategy to allow accumulation of lipid reserves to fuel metamorphosis and the non-feeding puerulus stage. Depletion of lipid reserves may therefore impair development during later larval stages.

3.5.5 Mass scaling

Larval development of most crustaceans is characterised by a rapid increase in body mass (Anger, 2001; Ikeda et al., 2011), which has a substantial influence on the physiological processes of any marine organism, particularly during larval

development (Bishop and Torres, 1999). In order to achieve comparisons in metabolic rate of other crustacean larvae and spiny lobster larval stages, the intraspecific comparison of the relationship between R_r and body mass of larvae were normalised to a study temperature of 23°C using a temperature coefficient (Q_{10}) of 2 (Anger, 2001). This comparison revealed that R_r in spiny lobster larvae is strongly related to body size, with a mass scaling exponent of 0.82, which is typical of most organisms, including other crustacean larvae (scaling exponent of 0.84). However, the R_r of other crustacean larvae (crustacean line) is significantly higher than for spiny lobster (lobster line) species (Fig. 3.5) (Ikeda et al., 2011). The lower R_r of spiny lobsters may be partially explained by the morphological and physiological differences in these species compared to other crustacean larvae such as later development of gills, extended larval phases, slower growth rates and consequently reduced energy demand (Vernberg and Costlow, 1966; Schatzlein and Costlow, 1978; Chu and Ovsianico-Koulikowsky, 1994). Additionally, many of these spiny lobster species may be less active swimmers than other crustacean larvae (Ikeda et al., 2011).

The individual R_r of early stage *S. verreauxi* phyllosoma also lie below the lower 95% CI belt of the lobster line, suggesting the R_r of early stage *S. verreauxi* is lower than other spiny lobsters. This may be partly explained by differences in methodology, as a number of previous studies did not compensate for the effects of transfer stress on $\dot{M}O_2$. Increased $\dot{M}O_2$ due to transfer stress are a common occurrence in respirometry studies (Cech, 1990). Measurements of $\dot{M}O_2$ prior to metabolic rates becoming stable (adaption phase) should therefore be excluded in order to accurately determine metabolic rate (Herrmann and Enders, 2000). In comparison to previous studies, the smaller volume chambers used in the present study restricted activity of phyllosoma, particularly the 6 ml chamber used in early

stage phyllosoma. In contrast, the much larger chambers (up to sixteen times) used in previous studies would allow phyllosoma to be more active. Increased activity levels due to respirometry techniques have been reported previously in crustaceans (Bridges and Brand, 1980). Increased activity also causes a reduction in the mass scaling exponent (Bridges and Brand, 1980), which may explain why the mass scaling exponent for the lobster line was lower compared to the scaling exponents for *S. verreauxi* (0.97 for LD animals and 0.90 for HD animals) and supports the suggestion of increased activity of phyllosoma in previous respiratory studies. The mass scaling exponents for both LD and HD *S. verreauxi* phyllosoma are also within the range that indicates favourable conditions, since values greater than 1.0 have been recorded for crustacean larvae subjected to environmental stressors such as extreme temperatures (Dawirs, 1983; Yagi et al., 1990) and salinity (Yagi et al., 1990).

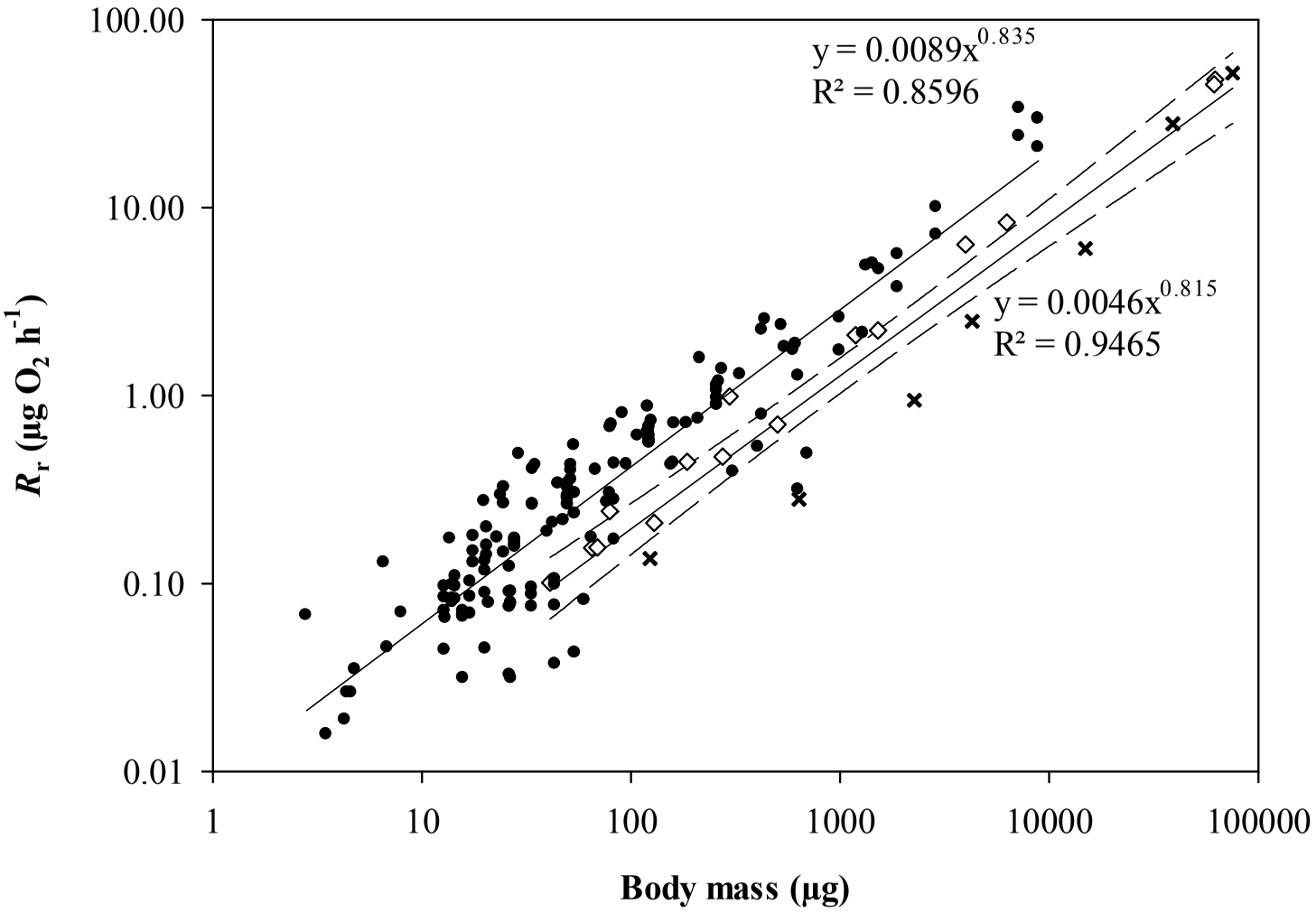
100.00
10.00
1.00
0.10
0.01
1
10
100
1000
10000
100000
R_r (µg O_2 h^{-1})
Body mass (µg)
y = 0.0089x^0.835
R² = 0.8596
y = 0.0046x^0.815
R² = 0.9465

Figure 3.5. Intraspecific comparison of the relationship between routine metabolic rate (R_r) and body mass of different crustacean larvae (●) (zoea I to megalopa spider crab, *Hyas araneus* (Anger and Jacobi, 1985; Anger, 1986; Anger et al., 1989b), stage I to V American lobster, *Homarus americanus* (Capuzzo and Lancaster 1979a; 1979b), zoea I to megalopa spider crab, *Hyas coarctatus* (Jacobi and Anger, 1985), zoea I to megalopa hermit crab, *Pagurus berhardus* (Dawirs, 1981; 1984; Anger et al., 1990), zoea I to megalopa tropical spider crab, *Libinia ferreirae* (Anger et al., 1989a), protozoea III to mysis III shrimp, *Metapenaeus ensis* (Chu and Ovsianico-Koulikowsky, 1994), zoea I to megalopa northern stone crab, *Lithodes maja* (Anger, 1996), nauplius III to mysis III pink shrimp, *Farfantepenaeus paulensis* (Lemos and Phan, 2001), zoea I to megalopa European shore crab, *Carcinus maenas* (Dawirs, 1983), zoea I to megalopa warmwater shrimp, *Caridina babaulti* (Idrisi and Salman, 2005), zoea I fiddler crab, *Uca thayeri* (Vernberg and Costlow, 1966; Belman and Childress, 1973), zoea I fiddler crab, *Uca rapax* (Vernberg and Costlow, 1966; Belman and Childress, 1973), zoea I fiddler crab, *Uca pugilator* (Vernberg and Costlow, 1966; Belman and Childress, 1973), zoea I fiddler crab, *Uca pugnax* (Vernberg and Costlow, 1966; Belman and Childress, 1973), zoea I to megalopa xanthid crab, *Rhithropanopeus harrisii* (Levine and Sulkin, 1979; Dawirs, 1984), megalopa Atlantic rock crab, *Cancer irroratus* (Sastry and McCarthy, 1973; Dawirs, 1984), megalopa mole crab, *Emerita talpoida* (Schatzlein and Costlow, 1978), zoea I to megalopa hermit crab, *Pagurus criniticornis* (Vernberg et al., 1981; Dawirs, 1984), zoea I to megalopa grapsid crab, *Chasmagnathus granulata* (Ismael et al., 1997), zoea I to megalopa bromeliad crab, *Metopaulias depressus* (Anger and Schuh, 1992), zoea I tanner crab, *Chionoecetes bairdi* (Incze and Paul, 1983), zoea I to II mole crab, *Emerita brasiliensis* (Moreira et al., 1981)). With spiny lobster species (◊) (puerulus western rock lobster, *Panulirus cygnus* (Lemmens, 1994b), instar 1 to 9 tropical rock lobster, *Panulirus ornatus* (Ikeda et al., 2011), instar 3 scalloped spiny lobster, *Panulirus homarus* (Ikeda et al., 2011), instar 1 California spiny lobster, *Panulirus interruptus* (Belman and Childress, 1973), instar 1 to 5 southern rock lobster, *Jasus edwardsii* (Bermudes et al., 2008), combined with *Sagmariasus verreauxi* (×) cultured at Low Density (LD) (this study) included for comparison. All R_r have been normalised to a study temperature of 23°C with a Q_{10}= 2 (Anger, 2001). Solid lines are linear regressions with corresponding 95% confidence intervals (dotted line) for spiny lobster species.

3.5.6 Conclusion

There is a clear shift towards higher protein catabolism in the final instar phyllosoma characterised by an increase in ammonia-N excretion rates and a decrease in the O:N ratio. This increased protein catabolism prior to metamorphosis is possibly due to the importance of lipid as an energy reserve for the non-feeding puerulus stage. Routine metabolic rate also increases in late stage phyllosoma, possibly due to the substantial physiological changes and high energy requirements of phyllosoma in preparation for the morphological changes associated with metamorphosis and increased swimming activity. The R_r of spiny lobster larvae under our experimental conditions was significantly lower than other crustacean larvae, which may be characteristic of their extended larval phase, slower growth rate, and larger body size. Although there were no significant differences in R_r and ammonia-N excretion rates of phyllosoma between LD and HD, the growth and development of larvae was negatively affected in the HD treatment possibly because of increased interactions between phyllosoma and prey.

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THE EFFECT OF HOLDING DENSITY ON GROWTH AND BIOCHEMISTRY DURING LARVAL ONTOGENY OF CULTURED SPINY LOBSTERS (*Sagmariasus verreauxi*)

Chapter IV

4.1 Abstract

Increased competition for available food at high stocking densities may restrict feed intake. Nutritional deficiencies caused by food deprivation will affect the competency of later stage lecithotrophic larvae. Biochemical analysis of phyllosoma during ontogeny provides information on the energy storage requirements of late stage phyllosoma and their ability to survive metamorphosis and achieve the energetic demands of the puerulus stage. This study examined the effects of stocking density on growth, development, and biochemical composition of the spiny lobster *Sagmariasus verreauxi* during ontogeny. Phyllosoma were cultured at two densities, with the High Density (HD) treatment (60 to 15 phyllosoma l^{-1}) being three times greater than the Low Density (LD) treatment (20 to 0.5 phyllosoma l^{-1}). Protein (% dry mass; DM) measured directly using the Lowry method was consistently lower than crude protein as calculated from nitrogen (N) content (% DM) using N ×6.25. This suggested that a conversion factor of 6.25 was too high and underestimated considerable amounts of non-protein nitrogenous compounds. Phyllosoma growth and development were more advanced in LD phyllosoma after 108 d. Instar 17 LD phyllosoma were also significantly larger (75.47 ± 4.18 mg DM) than instar 17 HD phyllosoma (63.69 ± 2.57 mg DM). Lipid and crude protein content (% DM) increased with phyllosoma development at both densities, with both components being significantly higher in instar 15 and 17 phyllosoma than instar 1 phyllosoma. Crude protein content (% DM) was not affected by density, however, lipid content (14.64 ± 1.35% DM) and the C:N ratio (4.16 ± 0.09) of LD phyllosoma were significantly higher than HD phyllosoma (13.53 ± 1.20% DM and 4.10 ± 0.08 for lipid content and the C:N ratio, respectively). The C:N ratio confirmed that

proportionally more lipid than protein was accumulated during larval development before a significant increase in energy utilisation between instar 17 and the puerulus stage. This increased energy utilisation was characterised by a more than 21% decrease in lipid reserves that fuelled the process of metamorphosis and the non-feeding puerulus stage. The difference in growth, development, and biochemical composition of phyllosoma was possibly due to a reduction in feeding success and increased energy expenditure of HD phyllosoma. Larval culture should therefore avoid high stocking densities to allow phyllosoma to accumulate the energy reserves required to successfully complete metamorphosis.

4.2 Introduction

The survival and recruitment success of spiny lobster species depends on the efficient utilisation of energy from both food and body reserves accumulated during earlier larval stages (Capuzzo and Lancaster, 1979; Sasaki et al., 1986). In *Sagmariasus verreauxi*, ontogeny consists of lecithotrophic and planktotrophic stages, and a shift from a planktonic to a benthic lifestyle following metamorphosis. This is accompanied by many changes in anatomy, morphology, and biochemical composition, which often result in different strategies of energy utilisation (Lovett and Felder, 1989; Dall et al., 1990). However, while the substrates for energy utilisation have been well documented for several decapod species during ontogeny (Anger et al., 1989; Anger and Harms, 1990; Lemos and Phan, 2001b), such studies are uncommon in spiny lobsters due to the difficulty of culturing phyllosoma through their long and complex larval phase (Ritar et al., 2003) or capturing undamaged individuals from the wild (Ikeda et al., 2011).

After hatching, phyllosoma progress through a series of planktonic larval instars which are characterised by changes in morphology and increased size, and each instar is clearly separated by moulting (Anger, 2001). The planktonic larval phase may take up to 12 months for *S. verreauxi* in the wild (Booth and Phillips, 1994) or 8 months in the laboratory, with 11 distinct morphological stages and 17 moults (Kittaka et al., 1997). Since larvae undergo intermittent growth and development achieved by each successive moult, changes in energy utilisation and biochemical composition may result from shifts in feeding behaviour and diet (Omori, 1979; Ikeda, 1984; 1985; Matsuda et al., 2009). Accordingly, shifts in energy substrate utilisation may provide information on changes in the nutritional

requirements of phyllosoma (Sasaki et al., 1986). The high metabolic rates of larvae and rapid biochemical turnover means they are particularly susceptible to any nutritional deficiencies caused by food deprivation (Jones et al., 1997) incurred at high stocking densities.

At high stocking densities, increased competition for available food may restrict feed intake (Minagawa and Murano, 1993; Mikami, 1995). Reduced feeding success at high density may also affect the viability of phyllosoma in later stages and their ability to survive metamorphosis (Smith and Ritar, 2006). Larval rearing of crustaceans often uses high densities but without testing the effects on physiological parameters (Emmerson and Andrews, 1981). High stocking density may also contribute to excessive expenditure of energy caused by increased physical interactions with prey and other phyllosoma such as, entanglement or physical damage of phyllosoma, which may impair their ability to actively capture prey (Mikami, 1995; Smith and Ritar, 2006). Larvae cultured under optimal conditions should develop through the shortest possible number of instars, whereas weak or stressed larvae may develop through a higher number of instars and have extended moult increments (Anger, 2001). Moult cycle duration and moult increment are genetically set at an optimal level, which is then modified by nutrition and other extrinsic factors (Freeman, 1990). Because larvae increase in biomass during their development, successive stages also require higher amounts of energy per individual (Anger, 2001).

Factors affecting biochemical composition of crustacean larvae have been studied extensively in a large number of species (Conover, 1978; Anger, 2001; Lemos and Phan, 2001b; Ritar et al., 2003). Most available information is based on biochemical (protein, lipid, and carbohydrate) and elemental (carbon; C, hydrogen; H,

and nitrogen; N) composition of decapod crustaceans (Childress and Nygaard, 1974; Ikeda and Bruce, 1986; Ikeda and Skjoldal, 1989). However, very few studies have analysed biochemical and elemental composition during the entire larval period (Omori, 1979). Variations in individual biochemical components during ontogeny reflect the dynamic balance between nutrient acquisition and metabolism, and identifying shifts in substrate utilisation are essential when determining the components conserved as energy reserves and those catabolised (Olsen, 1998). Changes in the relative proportions of lipids, proteins and carbohydrates in the overall biomass have been frequently used as indicators of larval viability, which may be correlated with the overall survival (Ferron and Leggett, 1994; Suthers, 1998).

Lipids are essential nutrients (Kattner et al., 1994); they provide essential fatty acids, cholesterol and a large amount of energy. Lipids are the primary energy reserve accumulated during larval development of crustaceans (Heras et al., 2000) and are metabolised by the non-feeding puerulus stage in spiny lobsters (Jeffs et al., 2001). The relationship between reduced growth and reduced lipid retention (Cockcroft, 1997; Jeffs et al., 1999; Ward et al., 2003) highlights the importance for further studies on factors that affect lipid content of spiny lobsters. Protein accounts for the largest proportion of biomass in crustacean larvae (Pandian, 1967; Capuzzo and Lancaster, 1979; Anger et al., 1983; Anger, 1988). Whilst, metabolism of protein as an energy source has been well documented in many aquatic species (Claybrook, 1983; Finn et al., 1995; Lemos and Phan, 2001a), it has not been investigated in *S. verreauxi* phyllosoma. The efficiency with which protein is assimilated into new tissues is influenced by a number of factors, including quality and quantity of dietary protein, the presence of other energy substrates in the diet and the efficiency of nutrient digestion (Wilson, 1989; Carter and Houlihan, 2001; Ward et al., 2003).

Changes in biochemical composition during the entire course of phyllosoma development have rarely been measured (Ikeda et al., 2011). Also, simultaneous measurements of protein (measured directly) and crude protein (calculated from N), have not been investigated previously in phyllosoma. Therefore, the aim of the present study was to investigate the stage-specific changes in biochemical composition, with regard to lipid and protein content, during ontogeny of *S. verreauxi* in order to evaluate energy storage and utilisation through larval development and better understand the energy storage requirements of late stage phyllosoma prior to metamorphosis. Further, the effects of stocking density on biochemical composition and growth and development, measured as dry mass (DM), total length (TL), carapace width (CW), and instar stage, of phyllosoma was investigated through comparison of Low Density (LD) and High Density (HD) treatments.

4.3 Materials and methods

4.3.1 Experimental animals

Broodstock were fed a combination of fresh whole blue mussels (*Mytilus edulis*) and commercial prawn pellet (Higashimaru, Vital No. 12, http://www.k-higashimaru.co.jp/) twice a week. They were held in captivity in a 4,000 l fibreglass tank year round under a regime of ambient photoperiod and water temperature (11°C-19°C), 33-35 psu salinity, pH approximately 8.1, and 90-100% oxygen saturation at the Institute for Marine and Antarctic Studies (IMAS), Taroona, Hobart. Phyllosoma used in this experiment were hatched on the 8th of February 2009 from one female weighing approximately 2.0 kg.

4.3.2 Larval culture

Triplicate 7 ml sub-samples of newly-hatched phyllosoma were counted from a 20 l bucket to estimate density and then stocked into 50 l cylindrical tanks with flow-through seawater filtered to 1 µm and treated with ozone and ultra-violet irradiation according to Jensen et al. (2011) and maintained at 21-23°C using an industrial heat/chill unit (Carrier, C010PHH7AA, Australia). Four replicate tanks were initially stocked at LD (20 phyllosoma l^{-1}) and HD (60 phyllosoma l^{-1}). However, due to high mortalities in the LD treatment caused by an acute ozonation problem during instar 9, this treatment was reduced to three replicates in order to maintain experimental treatment densities. Because phyllosoma increased in size with successive larval stages, the densities were progressively reduced and the density of the HD treatment was three times greater than the LD treatment throughout the experiment. Stocking densities of the LD treatment were reduced to 10, 5, 2.5, 1.25,

and 0.5 l^{-1} at instars 3, 6, 9, 12, and 15, respectively, by randomly culling phyllosoma. Stocking densities of the HD treatment were reduced to 30, 15, 7.5, 3.75, and 1.5 l^{-1} at equivalent instars.

Phyllosoma were fed a combination of freshly prepared blue mussel (*M. edulis*) gonad (~ 5 mm diameter) once daily and on-grown *Artemia* of 5.0 - 8.0 mm in length three times a week. Despite extensive water treatment, the daily addition of food increased bacterial levels and tanks (50 l) were cleaned every 14 days to reduce accumulation of bacteria and fouling.

4.3.3 Feed production

Artemia nauplii were stocked in 670 l tanks at 5 *Artemia* ml^{-1} and on-grown to approximately 8.0 mm in flow-through tanks receiving a diet of blended brine shrimp food (consisting of rice pollard, soyflour and wheat flour; Eyre Peninsula Aquafeeds Pty Ltd, South Australia) and algae (T. *Isochrysis* and *Chaetoceros muelleri*). Prior to feeding to phyllosoma, gut bacteria in *Artemia* were minimised by purging in dense cultures of *C. muelleri* (10^7 cells ml^{-1}), as described by Tolomei et al. (2004), and 400 ppm formalin for 30 min before rinsing with filtered seawater and feeding to phyllosoma culture tanks. *Artemia* feeding rates were based on phyllosoma density and kept consistent between treatments with the HD treatment receiving three times more (1.38 g DM) than the LD treatment (0.46 g DM). The length:mass relationship of *Artemia* cultured at IMAS was used to determine DM of ration based on *Artemia* length. Uneaten *Artemia* remaining from the previous meal were flushed from the tanks prior to the next meal of *Artemia*.

Meals of fresh pieces (5.0 mm in diameter) of blue mussel (*M. edulis*) were kept consistent between treatments (0.25 ml $phyllosoma^{-1}$) with the HD treatment

receiving three times more mussel than the LD treatment. Uneaten mussel was siphoned from culture vessels each day before feeding the next meal of mussel.

4.3.4 Biochemical composition

Biochemical composition of phyllosoma from both HD and LD treatments were determined at instars 1, 6, 9, 12, 15, 17, and the puerulus stage. A sample of phyllosoma (n= 20) from each tank was measured at regular intervals for TL and CW using a profile projector (Nikon 6C, Japan) and staged according to Kittaka et al. (1997) under a dissecting microscope (AIS Optical) at 10-40x magnification to determine intermoult periods. Phyllosoma for biochemical analyses were obtained from the initial hatch population at instar 1 and from random culling of animals when densities were reduced for each treatment at instars 6, 9, 12, 15, and 17. All individuals that progressed to the puerulus stage in the LD treatment were used for biochemical analyses. No animals were advanced enough to progress to the puerulus stage in the HD treatment. Prior to sampling, phyllosoma were starved for 24 h to clear the digestive tract of food and faeces. They were then counted and rinsed in distilled water to remove external salts and blotted dry with paper towel. Phyllosoma and pueruli from each tank were placed into pre-weighed aluminium foil pockets and stored at -80°C until further analysis. Samples were weighed to the nearest 10 µg on a precision balance (Mettler AT261 DeltaRange, Metler-Toledo, Switzerland), freeze-dried (Dynavac FD3, Sydney), re-weighed to calculate sample DM and then ground to a homogenous powder with a glass mortar and pestle. Individual DM was calculated for instars 6 to 17 by dividing the sample DM by the number of phyllosoma in the sample. Biochemical composition was determined from the mean of all replicates in LD and HD treatments.

Total lipid was analysed using a modified Bligh and Dyer (1959) one-phase methanol:chloroform:water extraction (10:20:8, by volume). Each sample was extracted overnight and the phases were separated the following day by addition of chloroform and water (final solvent ratio, 1:1:0.9, v/v/v, methanol:chloroform:water). The total solvent extract was concentrated using rotary evaporation at 40ºC and lipid content was determined by mass.

Total C, H, and N were measured by a flash elemental analyser (Thermo Finnigan EA 1112 Series) at the Central Science Laboratory, University of Tasmania, Hobart. Crude protein was calculated as N multiplied by 6.25. Total protein was determined by a modified Lowry et al. (1951) method (Houlihan et al., 1986), using approximately 20 mg of dry tissue. All biochemical analyses were performed using analytical duplicates.

4.3.5 Data analysis

Residual plots were used to explore normality and homogeneity of data. Percentage data were arcsine transformed prior to further statistical analysis. Differences in mean biochemical composition between densities and instar stage were compared using two-way analysis of variance (ANOVA) and significant differences identified using Tukey's HSD tests for post-hoc multiple comparisons. Where no interaction between two factors occurred, differences between densities or instars were reported. Instar 9, 12, and puerulus samples are presented on graphs but were excluded from analyses because there was insufficient replication (no LD replicates for instar 9 and 12 and no HD replicates for pueruli, resulting in unbalanced analyses). Growth measurements were compared between densities using an independent sample t-test and where no significant differences occurred two-way ANOVA is presented. Exponential regression was used to describe the relationship between growth

parameters and instar stage. Linear and power regression was used to analyse the relationship between growth parameters and culture day using SigmaPlot version 12 (2011 Systat Software Inc.). ANOVA and t-tests were performed using SPSS version 16.0 (2007 SPSS Inc.). The level of significance for all analyses was determined at $P<0.05$. Data are presented as mean ± standard error (SE) unless stated otherwise.

4.4 Results

4.4.1 Growth

The individual DM, TL, and CW of phyllosoma increased exponentially with development for both LD and HD treatments (Table 4.1, Fig. 4.1). There was no significant difference in DM of phyllosoma between densities up to instar 15 (F= 0.497, df 1, 99, *P*= 0.483). However, phyllosoma in the LD treatment were significantly heavier than those in the HD treatment at instar 17 (t= -2.400, df 22, *P*= 0.025, Fig. 4.1A). Total length (two-way ANOVA; F= 0.768, df 1, 99, *P*= 0.383) and CW (two-way ANOVA; F= 0.628, df 1, 99, *P*= 0.430) were not significantly different between density treatments when compared at instar development stage (Table 4.1). However, density affected the rate of growth and development of phyllosoma, where TL (two-way ANOVA; F= 87.473, df 1, 61, *P*< 0.001, Fig. 4.2A), CW (two-way ANOVA; F= 90.793, df 1, 61, *P*< 0.001, Fig. 4.2B) and instar stage (two-way ANOVA; F= 72.26, df 1, 61, *P*< 0.001, Fig. 4.2C) were significantly greater in the LD treatment after 108 d in culture.

4.4.2 Biochemical composition

There was no significant interaction between density and instar for percentage lipid content of phyllosoma (two-way ANOVA; F= 1.079, df 3, 32, *P*= 0.377). However, density (two-way ANOVA; F= 23.798, df 1, 32, *P*< 0.001) and instar stage (two-way ANOVA; F= 635.027, df 3, 32, *P*< 0.001) both had a significant effect on lipid content of phyllosoma (Fig. 4.3A). The lipid content of LD phyllosoma (14.64 ± 1.35% DM) was significantly higher than HD phyllosoma (13.53 ± 1.20% DM). The lipid content of phyllosoma was also significantly higher at instars 15 and 17

compared to instar 1 and 6 phyllosoma at both densities. The lipid content of LD pueruli (15.31 ± 0.26% DM) dropped by over 21% compared to content at instar 17 (19.50 ± 0.35% DM) immediately after metamorphosis.

There was no significant interaction between density and instar for percentage crude protein content of phyllosoma (two-way ANOVA; F= 0.775, df 3, 32, *P*= 0.519). Density also did not significantly affect the percentage crude protein content of phyllosoma (two-way ANOVA; F= 0.040, df 1, 32, *P*= 0.844). However, instar stage had a significant impact on crude protein content of phyllosoma (two-way ANOVA; F= 117.044, df 3, 32, *P*< 0.001, Fig. 4.3B). The crude protein content of instar 1 phyllosoma was significantly lower than all other instars at both densities. Crude protein represented the largest percentage of DM in phyllosoma at both densities and was highest in pueruli (58.32 ± 1.16% DM), which was nearly 11% higher than instar 17 phyllosoma (52.62 ± 0.16% DM) in the LD treatment.

There was no significant interaction between density and instar for the C:N ratio in phyllosoma (two-way ANOVA; F= 1.625, df 3, 32, *P*= 0.210). However, density (two-way ANOVA; F= 13.569, df 1, 32, *P*= 0.001) and instar stage (two-way ANOVA; F= 646.953, df 1, 32, *P*< 0.001) both had a significant effect on the C:N ratio of phyllosoma (Fig. 4.3C). The C:N ratio of LD phyllosoma (4.16 ± 0.09) was significantly higher than HD phyllosoma (4.10 ± 0.08). The C:N ratio was also significantly different between all instars analysed at both densities and increased significantly with each successive instar, ranging between 3.66 ± 0.00 for instar 1 phyllosoma and 4.46 ± 0.02 for instar 15 phyllosoma, before decreasing between instars 15 and 17. There was also a sharp decrease by over 10% in the C:N ratio between instar 17 and the puerulus stage in the LD treatment.

There was no significant interaction between density and instar stage for the conversion factor of N to protein (two-way ANOVA; F= 0.301, df 3, 16, *P*= 0.824, Table 4.2). The conversion factor estimated from the relationship between N and protein was also not significantly different between densities (two-way ANOVA; F= 0.211, df 1, 16, *P*= 0.658). However, the conversion factor was lower than 6.25 for both densities, ranging between 3.01 ± 0.17 and 5.04 ± 0.17. Instar stage also had a significant impact on the conversion factor at both densities (two-way ANOVA; F= 52.739, df 3, 16, *P*< 0.001). The conversion factor of N to protein for instar 1 phyllosoma was lower than all other instars.

Table 4.1. Details of exponential ($y= ae^{bx}$) regressions describing the relationship between instar development stage (instar) and dry mass (DM), total length (TL), and carapace width (CW), linear ($y= ax + b$) regressions describing the relationship between age (day) and total length (TL), and carapace width (CW), and power ($y= a*x^{b}$) regressions describing the relationship between age (day) and mean instar stage of *Sagmariasus verreauxi* phyllosoma cultured at Low Density (LD) or High Density (HD) presented in Figs. 4.1 and 4.2.

		Density	a	b	R^2	df	F	*P*
Instar	Dry mass (mg)	Low	0.273	0.331	0.99	6	15603.74	<0.001*
		High	0.624	0.274	0.99	6	553.97	<0.001*
	Total length (mm)	Low	2.451	0.158	0.99	6	3601.92	<0.001*
		High	2.602	0.152	0.99	6	1753.06	<0.001*
	Carapace width (mm)	Low	1.613	0.148	0.99	6	1540.45	<0.001*
		High	1.660	0.145	0.99	6	772.29	<0.001*
Day	Total length (mm)	Low	0.136	0.608	0.98	7	266.18	<0.001*
		High	0.113	1.476	0.99	7	626.73	<0.001*
	Carapace width (mm)	Low	0.079	0.472	0.98	7	351.32	<0.001*
		High	0.066	0.946	0.99	7	563.70	<0.001*
	Mean instar	Low	0.430	0.691	0.99	7	1099.48	<0.001*
		High	0.577	0.618	0.99	7	533.41	<0.001*

* indicates significant regression ($P<0.05$).

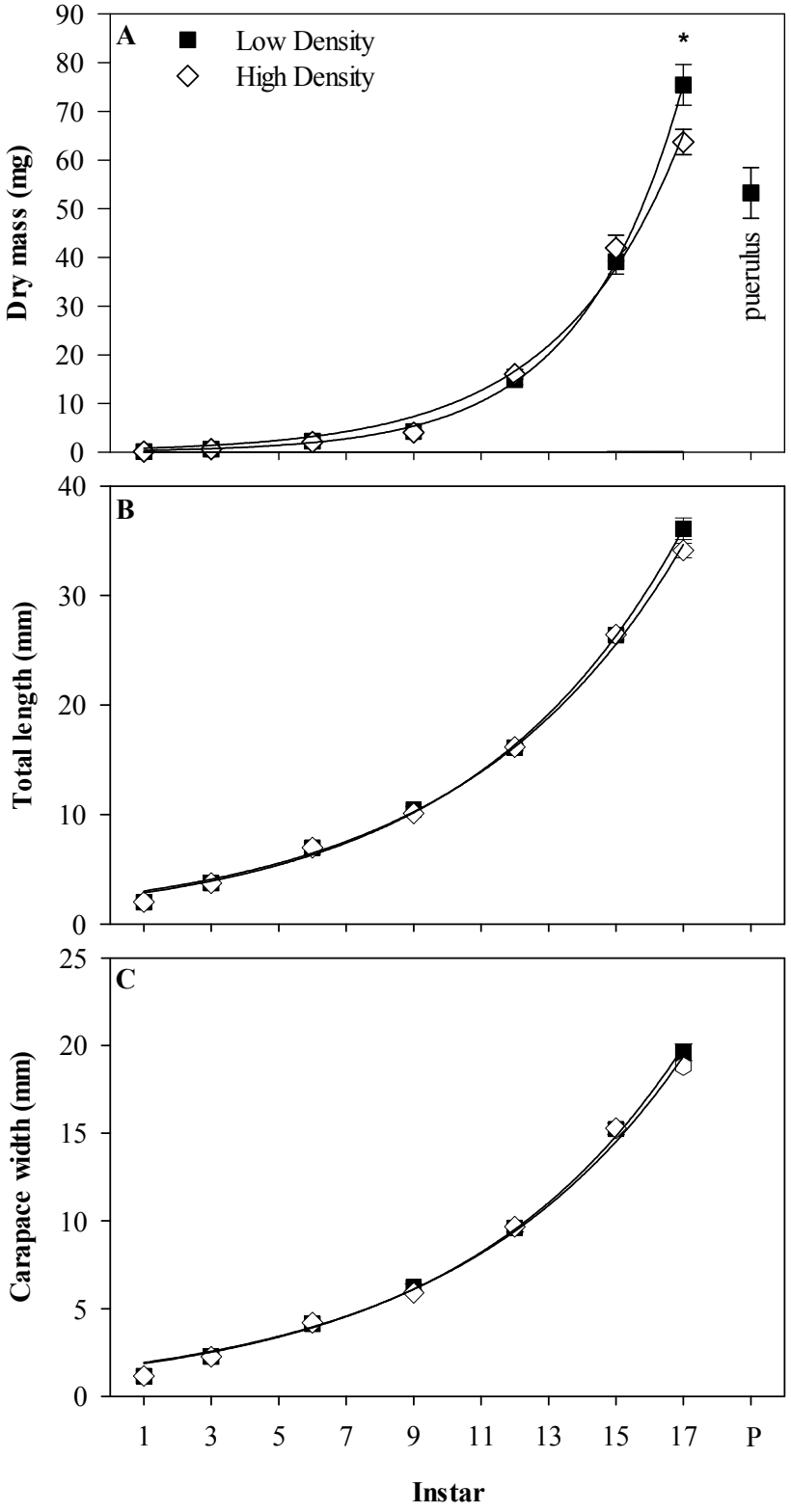

Figure 4.1. Relationship between: (A) dry mass (DM); (B) total length (TL); (C) carapace width (CW) and instar development stage of *Sagmariasus verreauxi* phyllosoma cultured at Low Density (LD) or High Density (HD). P denotes puerulus stage. Plots bearing asterisks are significantly different between densities at the instar indicated ($P<0.05$). Values are mean (±SE). Details of regressions are given in Table 4.1.

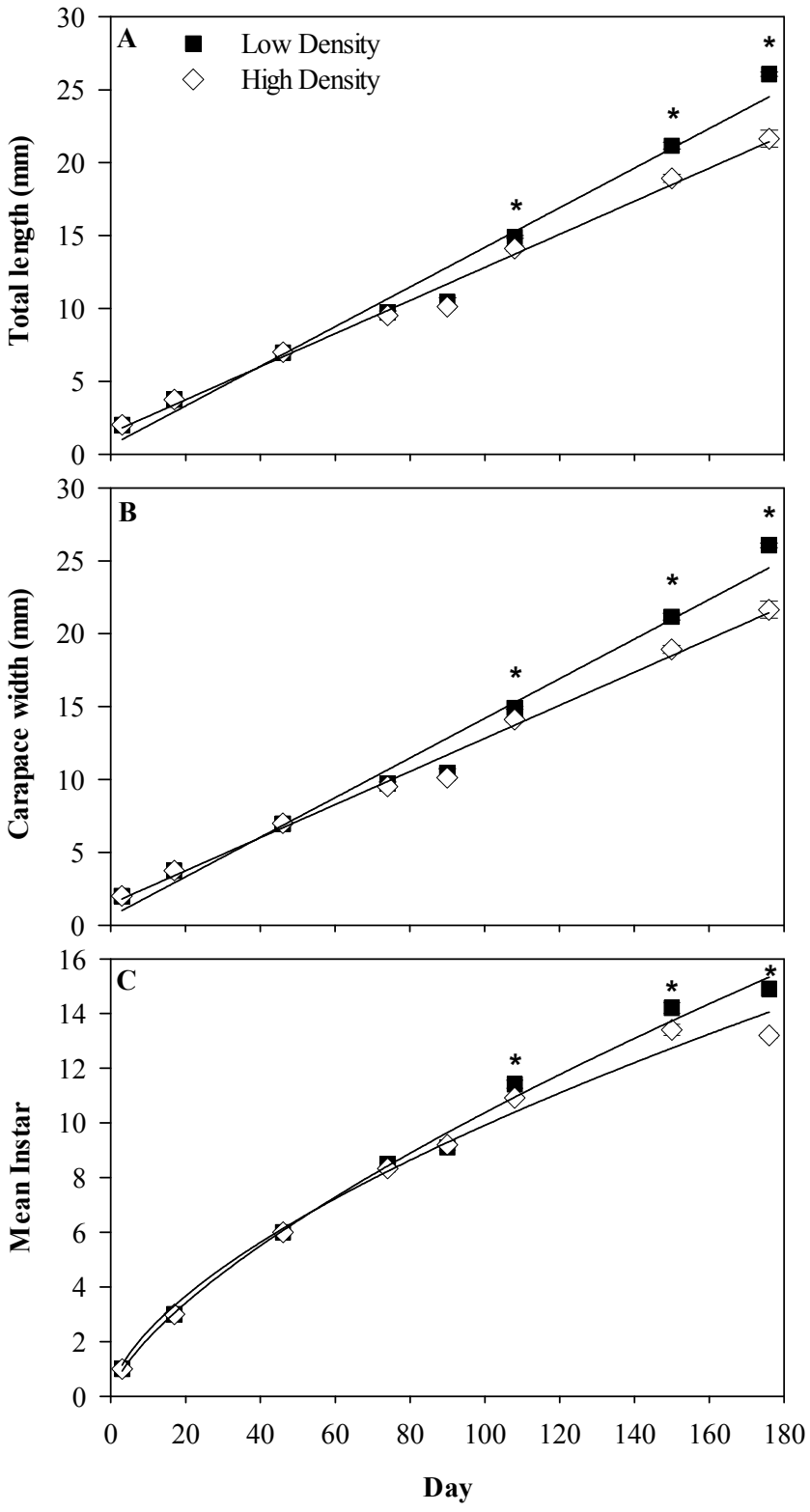

Figure 4.2. (A) Total length (TL); (B) carapace width (CW) and; (C) mean instar stage of *Sagmariasus verreauxi* phyllosoma at different days from hatch when cultured at Low Density (LD) or High Density (HD). Plots bearing asterisks are significantly different between densities (P<0.05). Values are mean (±SE). Details of regressions are given in Table 4.1.

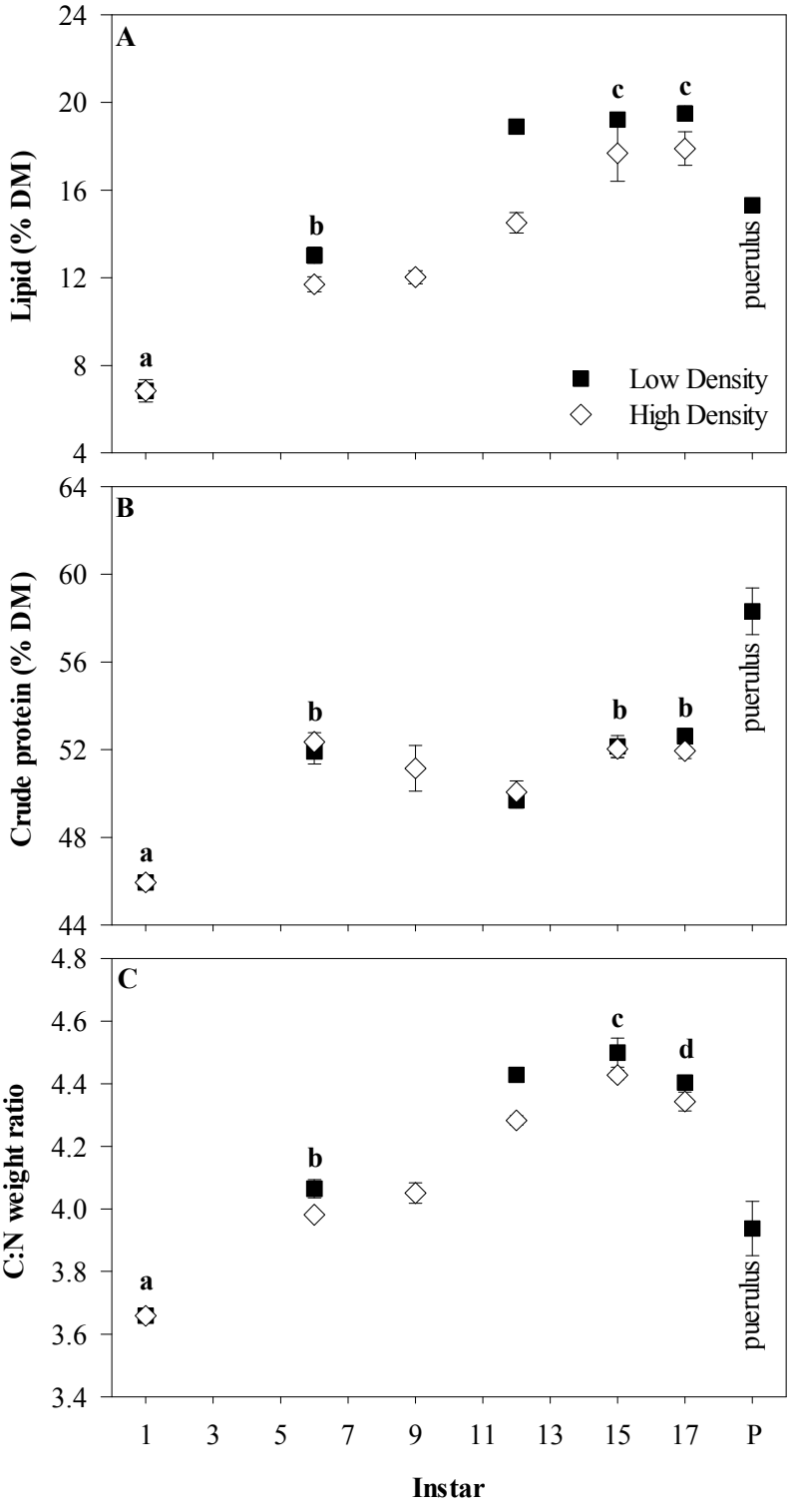

Figure 4.3. Developmental changes in: (A) lipid content (% dry mass; DM); (B) crude protein (% dry mass; DM) based on a conversion factor of 6.25 from N content and; (C) atomic C:N ratio of *Sagmariasus verreauxi* phyllosoma cultured at Low Density (LD) and High Density (HD). Data points bearing different superscripts are significantly different between instar stages ($P<0.05$). The lipid content and C:N ratio of Low Density (LD) phyllosoma are significantly higher than High Density (HD) phyllosoma ($P<0.05$). Instar 9, 12, and puerulus measurements were excluded from statistical analysis. P denotes puerulus stage. Values are mean (±SE).

Table 4.2. The protein, elemental nitrogen (N) (±SE), difference in N (between elemental analysis and based on a conversion factor of 6.25 from the protein content), percentage non-protein N, and conversion factor of N to protein (±SE) based on percent of dry mass (% DM) of *Sagmariasus verreauxi* phyllosoma cultured at Low Density (LD) and High Density (HD) from hatch to instar 15.

Instar	Density	Protein (%)	N (%)	Difference in N (%)	Non-protein N (%)	Conversion factor
1	Low	22.10	7.35 ± 0.02	3.82	51.90	3.01 ± 0.17^{a}
1	High	22.10	7.35 ± 0.02	3.82	51.90	3.01 ± 0.17^{a}
6	Low	38.15	8.09 ± 0.09	1.98	24.51	4.72 ± 0.17^{b}
6	High	39.68	8.52 ± 0.07	2.18	25.52	4.66 ± 0.17^{b}
12	Low	39.55	7.85 ± 0.00	1.52	19.40	5.04 ± 0.17^{b}
12	High	39.07	8.12 ± 0.10	1.87	23.00	4.81 ± 0.17^{b}
15	Low	39.35	8.42 ± 0.09	2.12	25.21	4.67 ± 0.17^{b}
15	High	40.10	8.45 ± 0.07	2.03	24.06	4.75 ± 0.17^{b}

Letter superscripts indicate significant differences ($P<0.05$).

4.5 Discussion

4.5.1 Growth

In the present study, phyllosoma in the LD treatment were larger and more advanced in regard to instar stage compared to HD phyllosoma. This is consistent with Smith and Ritar (2006) who found that growth and survival of *Jasus edwardsii* phyllosoma was significantly reduced at densities above 40 l^{-1}. Smith and Ritar (2006) also noted that excessive clumping of phyllosoma in high density cultures could impair their ability to actively capture prey and reduce feed intake. This is due to phyllosoma in high density cultures becoming entangled, causing physical damage to limbs. Phyllosoma that sustain physical damage during moulting may also acquire moult deformities, which results in extended instar durations and usually death at the following moult (Smith and Ritar, 2006). The reduced size and development of phyllosoma in the HD treatment may have resulted from reduced feeding success and higher energy expenditure caused by increased interaction between phyllosoma, which may affect their ability to survive and successfully complete metamorphosis. This is consistent with Emmerson and Andrews (1981) who found development time increased as a function of stocking density in *Penaeus indicus* and survival and growth decreased with increasing density.

4.5.2 Biochemical composition

Instar stage had a significant impact on the biochemical composition of *S. verreauxi* during larval development. Changes in energy utilisation between individual instars are a major cause of biochemical variation in larvae and, therefore, are an important consideration in any crustacean larval study (Anger and Harms,

1990). Since crustacean larval growth is a discontinuous process achieved by successive moults (Thomas et al., 2003) energy costs to meet the demands of moulting may vary considerably with development and culture conditions (Omori, 1979; Ikeda, 1984). As size increases, the moult interval generally increases and allows crustaceans to accumulate the energy reserves required for the next moult (Thomas et al., 2003). There is a considerable increase in biomass of individual phyllosoma and, consequently, of all biochemical components during larval development of *S. verreauxi*. The rate at which individual biochemical components are accumulated may indicate the most important energy reserves required during larval development to allow constant moulting.

Lipids play a major role as energy reserves during growth and development of crustacean larvae (Holland, 1978). In the present study, the large reduction in lipid content of pueruli by over 21% from instar 17 indicates that *S. verreauxi* phyllosoma utilise substantial amounts of lipid as an energy source during metamorphosis. It is well documented that phyllosoma preferentially store lipid for metabolic processes (Johnston et al., 2004), and high lipid levels have even been suggested as a possible cue for metamorphosis in some spiny lobster species by reaching a critical nutritional point (McConaugha, 1982; McWilliam and Phillips, 1997). Previous studies have also found the puerulus migration of *J. edwardsii* is fuelled by lipid reserves built up over the long larval period to allow them to actively swim the large distances across the shelf to settle inshore (Jeffs et al., 1999; 2001). The characteristic long larval period of spiny lobsters may therefore be necessary to allow phyllosoma to reach a critical level of stored energy reserves to fuel metamorphosis and puerulus migration.

High lipid levels in phyllosoma may be associated to their planktonic lifestyle, as many planktonic crustaceans tend to store lipid due to its high energy content, low

density, and positive buoyancy (Chu and Ovsianico-Koulikowsky, 1994). Jeffs et al. (2001) suggested phospholipid was the major lipid class stored by *J. edwardsii* phyllosoma possibly due to its characteristic transparency, which is critical during the planktonic life stage when phyllosoma are extremely vulnerable to pelagic visual predators. The ability of phyllosoma to preferentially store lipid may therefore be an essential physiological survival strategy to allow accumulation of energy reserves. This strategy is not unique to spiny lobsters and also occurs in other crustaceans and aquatic animals. Sasaki et al. (1986) found that newly settled *Homarus americanus* depend on stored lipid reserves during transformation from a planktonic to benthic life phase while they are adjusting to a new feeding behaviour and benthic habitat.

Cultured phyllosoma typically contain between 7 and 21% lipid (Nelson et al., 2003; Ritar et al., 2003). This is similar to the 6.8 (instar 1) to 19.5% DM (instar 17) lipid range observed in the present study and reflects the extent of lipid accumulation during culture. Wild phyllosoma, however, contain up to 35% DM lipid (Phleger et al., 2001), which suggests this is the amount required for successful completion of the larval phase. The difference in energy-rich lipid reserves between wild and cultured phyllosoma may be attributed to the lack of prey diversity encountered by cultured phyllosoma. Wild phyllosoma feed on a variety of prey including diatoms, dinoflagellates and zooplankton such as cnidarian jellies, which provide phyllosoma with quantitatively and qualitatively diverse energy and nutrient sources (Phleger et al., 2001). Johnston et al. (2004) suggested that a lack of shifts in digestive enzyme activity in cultured phyllosoma fed a constant diet indicated an inability to accommodate shifts in their nutritional requirements compared to wild phyllosoma. It is therefore possible that phyllosoma are incapable of accumulating adequate amounts of energy when fed predominantly on *Artemia*, which adversely affects the growth

and development of cultured phyllosoma (Johnston et al., 2004). This suppressed growth has, however, also been observed in other decapods when cultured on their normal wild diet (Anger, 2001). Reduced growth of cultured phyllosoma could therefore also possibly be attributed to biotic and abiotic factors, including high densities associated with culture conditions and relatively high temperatures, because wild phyllosoma are widely dispersed and inhabit cooler waters at certain times of the year (Booth, 1994). Future research should also investigate the maternal effects of broodstock feeding on different diets to determine if this has an influence on phyllosoma quality, particularly their ability to accumulate energy reserves.

4.5.3 C:N ratio

Protein (46 – 58% DM) constituted the largest proportion of DM in *S. verreauxi* phyllosoma compared with lipid (7 – 20% DM) at both densities. The relative proportions of protein and lipid in the present study were similar to those observed previously in cultured *J. edwardsii* phyllosoma (Phleger et al., 2001; Ritar et al., 2003) and other crustacean larvae (Anger, 1998). The quantities of C and N are strongly associated with the amounts of total lipid and protein, respectively, and are standard measures of growth and chemical composition in the literature (Vinogradov, 1953; Beers, 1966; Platt et al., 1969; Ikeda, 1974; Omori, 1978; Gorsky et al., 1988; Hessen and Lyche, 1991). The C:N ratios (3.66 – 4.50) recorded in the present study are similar to those observed previously during larval development of *Panulirus cygnus* (Lemmens, 1994) and *Panulirus ornatus* (Ikeda et al., 2011). The C:N ratio for *S. verreauxi* increased during larval development up to instar 15 before a decrease at instar 17 and puerulus stage, indicating that protein becomes less important as an energy reserve in late phyllosoma and puerulus stages. Decreased importance of protein is probably a response by late stage phyllosoma to consume a larger

proportion of prey high in lipid such as krill in the wild (Nichols et al., 2001; Jeffs et al., 2004) and lipid-rich *Artemia* in culture (Johnston et al., 2004). The increased accumulation of lipid by late stage phyllosoma provides further evidence that lipids are important energy reserves for the non-feeding puerulus stage (Jeffs et al., 2001; Phleger et al., 2001).

Since pueruli do not feed they lose biomass during this stage due to depletion of energy reserves. This loss of biomass is similar to that observed in lecithotrophic crab megalopae (Anger, 1989). All other larval stages exhibit a significant increase in lipid and protein, reflecting a continual increase in energy storage with development. This continual increase in energy storage is necessary to meet the metabolic requirement of increasing biomass (Johnston et al., 2004).

Despite the considerable depletion of lipid reserves between instar 17 and the puerulus stage, the degree of this depletion does not appear to be correlated with metamorphosis success. Jeffs et al. (2001) found that successful metamorphosis in *J. edwardsii* was not likely a result of reaching a critical level of stored energy due to the variability in lipid reserves (21 to 39.5% DM) observed in new pueruli. Instar 17 phyllosoma with higher lipid contents did, however, metamorphose to larger pueruli (both carapace length and DM) (Jeffs et al., 2001). Depletion of lipid reserves during metamorphosis may also impact survival to the juvenile stage, because pueruli with higher lipid content have an increased swimming range to reach inshore settlement grounds (Jeffs et al., 1999; Jeffs et al., 2001). Reducing the depletion rate of lipid reserves by pueruli may therefore be a critical factor for improving lobster hatchery rearing success.

4.5.4 Protein and nitrogen

Stoichiometric relationships between elemental and biochemical components of biomass have commonly been used to convert data from one to the other (Gnaiger and Bitterlich, 1984). In particular, crude protein values are calculated from elemental N often using a conversion factor of 6.25 (Anger, 2001; Choa et al., 2010). The conversion factor of 6.25 is based on and tends to overestimate animal protein (Gnaiger and Bitterlich, 1984). As a consequence, protein values obtained from a modified Lowry et al. (1951) method in the present study were consistently lower than those calculated from the theoretical factor of 6.25. This was also found in several other crustacean studies comparing independent measurements of N and protein that suggested a conversion factor of 6.25 was probably too high (Childress and Nygaard, 1974; Anger et al., 1989; Anger and Harms, 1990). The conversion factors (3.01 – 5.04) of N to protein in the present study were also within the range (2.00 – 6.21) reported previously in a large variety of crustacean larvae (Anger et al., 1989; Anger and Harms, 1990) and is generally more typical of animals (Gnaiger and Bitterlich, 1984). Conversion factors lower than 6.25 in crustaceans may be explained by high amounts of N being present in major components other than protein, for example chitin or free amino acids (Tucker, 1978). The initial low conversion factor in newly-hatched phyllosoma and higher conversion factors in later-stage phyllosoma in the present study supports this theory, because the proportion of the highly chitinous exoskeleton to muscle tissue is much greater in smaller crustacean larvae. Several authors have found levels of between 13 and 30% chitin in different juvenile crayfish species (Hornung and Stevenson, 1971; Speck et al., 1972; Anger et al., 1992) and 6% of chitin mass in most decapod larval stages is estimated as chitin-N (Spindler-Barth, 1976), which would account for a large amount of the

non-protein N in the present study. In the study by Anger et al. (1992), low conversion factors of 3.36 were found, even after the subtraction of chitin-N from total N, suggesting large amounts of non-protein N are still present. These results indicate there may have been considerable amounts of nitrogenous compounds other than protein in phyllosoma, or some protein-like substances were not detected using the Lowry et al. (1951) method in the present study. Estimates of not only chitin, but amounts of chitin-N in *S. verreauxi* phyllosoma may be required in future research before an accurate conversion factor of N to protein can be determined for this species. Predictions of biochemical composition from elemental analysis may therefore be used accurately, providing that they are restricted to the species being studied (Anger et al., 1983).

4.5.5 Conclusion

There was an accumulation of lipid reserves during larval development of *S. verreauxi* and a significant increase in lipid utilisation beyond instar 17, characterised by a decrease in the C:N ratio. This is due to the depletion of lipid as an energy reserve to fuel metamorphosis and the non-feeding puerulus stage. Independent measurements of N and protein also found protein values were consistently lower than crude protein values calculated from the theoretical factor of 6.25, suggesting that a conversion factor of 6.25 was too high. This may be due to considerable amounts of N being present in chitin and free amino acids in phyllosoma. Although there were no significant differences in the protein content of phyllosoma cultured at LD and HD, the lipid content, C:N ratio, growth and development of phyllosoma was negatively affected in the HD treatment possibly because of reduced feeding success and increased energy expenditure. Therefore, larval culture requires careful examination

of stocking densities to prevent situations of reduced phyllosoma condition and growth.

4.6 References

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METABOLIC RESPONSES TO HANDLING, FORCE FEEDING, EXERCISING TO EXHAUSTION, AND ANAESTHESIA OF SPINY LOBSTER *Sagmariasus verreauxi* JUVENILES

Chapter V

5.1 Abstract

Stress is a regular occurrence in crustacean aquaculture, however there is limited information on the physiological responses of spiny lobsters to stress. By measuring the aerobic scope of lobsters we can better understand their ability to respond to stressors and help to reduce disease susceptibility and mortalities. This study determined the aerobic scope of juvenile spiny lobsters *Sagmariasus verreauxi* in order to evaluate their capacity to respond to exhaustive exercise, force feeding, anaesthesia, and handling stress. Standard (R_s), routine (R_r) and maximum (R_{max}) metabolic rates were determined by measuring oxygen consumption rates ($\dot{M}O_2$) and compared to pre-treatment R_r levels following handling, anaesthesia with 2-phenoxyethanol, anaesthesia with 2-phenoxyethanol and force feeding with saline solution, anaesthesia with AQUI-S and force feeding with saline solution, and exercising to exhaustion using an intensive chase protocol. Oxygen consumption rate of lobsters was elevated for 2.50 ± 1.40 h following handling, most likely due to increased activity. Both anaesthetics reduced activity of lobsters but increased $\dot{M}O_2$ following anaesthesia indicated anaesthetics did not eliminate the physiological stress effects associated with force feeding and handling. Active metabolic rate (R_{active}) following exercise to exhaustion was 3.7 ± 0.5 times pre-treatment R_s, and remained above pre-treatment R_r for 10.17 ± 0.70 h. Ammonia-N excretion and atomic O:N ratios did not differ between treatments and revealed lipid based metabolism, indicating lobsters in all groups had fully recovered 24 h post-treatment. Overall, the results demonstrated that handling did not cause a large increase in $\dot{M}O_2$ and lobsters recovered rapidly from handling. Conversely, the increase in $\dot{M}O_2$ induced by force feeding and anaesthesia required a large proportion of the aerobic scope that could

otherwise be utilised for other physiological functions. Exercise to exhaustion resulted in a large increase in $\dot{M}O_2$ and extended recovery periods, which may reduce growth and increase disease susceptibility and the risk of predation. It is, therefore, recommended that aquaculture handling procedures make precautions not to increase activity and exhaust lobsters and are carried out without anaesthesia to facilitate rapid recovery of lobsters. Force feeding may also provide an improved physiological method to previous feeding protocols if metabolic responses to anaesthesia are accounted for.

5.2 Introduction

During the course of an animal's life its metabolic rate will fluctuate between lower and upper extremes. The upper limit of aerobic metabolic rate is commonly referred to as the active metabolic rate (R_{active}) (Priede, 1985; Jobling, 1994). The lower limit is the maintenance metabolic rate, which is termed standard metabolic rate (R_s). The R_s is defined as the metabolic rate of an inactive animal in a post-absorptive nutritional state (Beck and Gropp, 1995). Factorial aerobic scope (FAS) is defined by dividing R_s from R_{active} of an individual and R_{active} is often estimated using chase protocols to exercise crustaceans to exhaustion (Booth and McMahon, 1992; Jimenez et al., 2008). Although R_{active} is often exercise induced, maximum metabolic rates (R_{max}) may also be induced by stressors such as unfavourable temperatures (White et al., 2008) and handling. During exercise, animals generally exhibit an increase in oxygen consumption rate ($\dot{M}O_2$) above R_s levels, which is referred to as excess post-exercise oxygen consumption (EPOC) (Lee et al., 2003). Information on the FAS is important because it represents the ability to perform all oxygen-consuming processes above that required for maintenance, and potentially, the capacity to respond to stressors (Killen et al., 2007). However, there is limited knowledge on the FAS of crustaceans due to the difficulties of accurately measuring R_s and R_{active} (Booth and McMahon, 1992). For example, many factors can increase $\dot{M}O_2$ above R_s levels (McMahon and Wilkens, 1983), while R_{active} can be under-estimated if the oxygen transport system of the animal is not fully exerted during exercise (Booth and McMahon, 1992). Therefore, it is important to standardise methodologies for defining R_s and R_{active} to avoid under or over-estimating these parameters.

Stress is a physiological response that alters the physiological state beyond the normal range as a consequence of intrinsic or extrinsic stimuli, which are referred to as stressors (Chrousos and Gold, 1992; Bonga 1997; Barton, 2002). Measurements of physiological changes associated with stress can give valuable information as to which stimuli are stressful and may be evaluated quantitatively by changes in $\dot{M}O_2$ (Lorenzon et al., 2007). Stressed lobsters typically have high $\dot{M}O_2$ and ventilation rates (Jouve-Dunhamel and Truchot, 1985). Stress can either be readily reversible if the physiological disruption is within the homeostatic capability of the animal, or it can be irreversible and eventually lead to death (Taylor et al., 1997). Stressors act in two ways; firstly, they create responses that threaten or disrupt the homeostatic equilibrium and they produce a series of behavioural and physiological responses considered to be compensatory or adaptive, allowing the animal to overcome the stress (Iversen and Eliassen, 2009). In the case of chronic stress, the animal may lose its adaptive ability and become dysfunctional, which may cause inhibition of growth, reproductive capacity, and reduce resistance to pathogens.

Stress is a regular occurrence in crustacean aquaculture, and Iversen and Eliassen (2009) identified handling, transport, drug administering, and confinement as potential stressors. In particular, handling animals is a common procedure in aquaculture, and has been shown to increase $\dot{M}O_2$ in lobsters (Taylor and Waldron, 1997) and prawns (Paterson, 1993). The characteristic response is to struggle to escape from capture. Excessive struggling can exhaust the animal and prolong the recovery period, and consequently, it is usually necessary to immobilise animals prior to handling (Ross and Ross, 1999). Anaesthetics are designed to allow handling of animals while reducing damage and physiological stress responses (Summerfelt and Smith, 1990). Several different compounds have been used for fish, but little is

known about the effects of these anaesthetics on lobsters. In order for an anaesthetic to be suitable for handling it should induce a state where the animal can maintain normal posture and gill movement and reduce the duration and magnitude of physiological stress responses (Davis and Griffin, 2004). Although it is known lobsters are stressed by handling, limited information is available on the physiological responses of lobsters to stress (Taylor et al., 1997). Changes in respiratory factors through air exposure during transport have been well documented for *Homarus gammarus* (Taylor and Whiteley, 1989; Whiteley and Taylor, 1990; 1992), *Jasus edwardsii* (Oliver and Morris, 1994; Taylor and Waldron, 1997) and *Panulirus argus* (Vermeer, 1987). These studies also include observations on recovery processes of lobsters during re-immersion. However, there is no information on stress responses of *Sagmariasus verreauxi* juveniles to handling, force feeding, anaesthesia and activity.

Studies on the effects of feeding on $\dot{M}O_2$, referred to as specific dynamic action (SDA) (Secor, 2009), in spiny lobsters are primarily limited by variations in individual ingestion rates due to leaching of diets and lobsters externally fragmenting diets before sweeping particles into the mouth (Sheppard et al., 2002). Previous physiological studies have determined lobster SDA responses by introducing meals to respirometry chambers and measuring $\dot{M}O_2$ after meals have been consumed (Crear and Forteath, 2000; 2001a; Perera et al., 2007; Kemp et al, 2009). However, these studies did not compensate for the uneaten diet due to leaching and the messy feeding behaviour of lobsters. Previous methods are also limited by the requirement to handle and manually open chambers to insert diets, which may cause the animal to become more active and increase $\dot{M}O_2$ at similar magnitudes as direct handling of the lobsters. Handling primarily affects $\dot{M}O_2$ of crustaceans by increasing their activity (Paterson, 1993), and consequently, respirometry trials usually exclude measurements during the

initial period following transfer to chambers (Herrmann and Enders, 2000; Steffensen, 2002). If measurements of $\dot{M}O_2$ are taken prior to metabolic rates stabilising (during the adaption phase) following handling or activity, these measurements may likely represent routine metabolic rate (R_r) and R_{active}, respectively, rather than R_s. Many animals also experience metabolic demands of activity and digestion simultaneously (Secor, 2009), which is attributed to the movement of limbs in lobsters to deliver food to the mouth during feeding. Specific dynamic action respiratory methodologies therefore require any activity, whether caused by handling of chambers and/or lobsters or feeding, to be accounted for to prevent over-estimating SDA responses. Anaesthesia and force feeding may provide an effective alternative to previous methods for ensuring all dietary components are ingested and by eliminating the increased activity associated with feeding.

The aim of the present study was to determine the aerobic scope of *S. verreauxi* juveniles in order to evaluate their capacity to respond to different stressors. The physiological responses to different stressors and anaesthetics were also investigated to provide valuable information for improving physiological research methodologies. The outcomes of this research may also help improve aquaculture handling and anaesthesia protocols to increase productivity and reduce mortalities.

5.3 Materials and Methods

5.3.1 Experimental animals

Juvenile *S. verreauxi* (310-370 mm carapace length, 314-513 g fresh mass) were reared from hatch at the Institute for Marine and Antarctic Studies (IMAS), Taroona, Hobart. Lobsters were held in captivity in a 4,000 l fibreglass tank from November to December under a regime of ambient photoperiod and 23°C water temperature and fed a combination of fresh whole blue mussels (*Mytilus edulis*) and commercial prawn pellet (Higashimaru, Vital No. 12, http://www.k-higashimaru.co.jp/) twice a week.

5.3.2 Experimental system

Juvenile lobsters were transferred from the holding tank and held individually in 40 l rectangular tanks under ambient photoperiod and 23°C water temperature with constant water flow. Tanks were covered with nets to prevent animals escaping. Artificial hides constructed from oyster mesh (5 mm mesh size) were placed into tanks to provide shelter. Individual lobsters were observed daily for moulting and then held until they reached the halfway point of their moult stage, as determined by the mean of their previous moult increments (around 78 d in lobsters of this size). The lobsters were fed a meal of half a blue mussel and left undisturbed for 72 h to ensure that all individuals were in the same post-prandial state before any measurements.

5.3.3 Treatment protocols

Lobsters were distributed into five treatment groups:

A. Handling (control)

B. Anaesthesia with 2-phenoxyethanol

C. Anaesthesia with 2-phenoxyethanol and force fed with saline solution

D. Anaesthesia with AQUI-S and force fed with saline solution

E. Swimming to exhaustion

Group A animals were removed from chambers and left undisturbed in a closed 15 l plastic container with 5 l of seawater for 15 min before being returned to the chamber. Group B animals were treated as Group A but were placed in 2% 2-phenoxyethanol (10 ml in 5 l) until animals did not avoid capture and the tail reflex was absent (<10 min). Group C animals were similarly anaesthetised with 2% 2-phenoxyethanol but were then force fed with 1% of their body mass (BM) of seawater using a polyethylene tube attached to a 5 ml syringe before being returned to respirometers. A red dye was added to the seawater solution to detect any regurgitation. Lobsters that rejected the seawater solution were excluded from analysis. Group D animals were anaesthetised and force fed according to Group C animals, but using AQUI-S as the anaesthetic. Group E animals were used to calculate R_{active} and were chased by hand in a 300 l tank until exhausted (<5 min) (exhaustion was defined as the point where animals did not avoid capture and the tail reflex was absent), before being returned to respirometers. Lobsters in all treatments were out of the chambers for the same time (15 min).

5.3.4 Oxygen consumption rate

Oxygen consumption rates were measured using an automated intermittent flow-through respirometer system. The lobsters were placed into 3.5 l respirometry chambers and allowed to acclimate for 16 h. Oyster mesh (5 mm mesh size) was fitted to the lower half of the chambers so that lobsters had traction on an otherwise

smooth surface and could remain quiescent (Dall, 1986). The chambers were immersed in a 300 l ambient tank (temperature bath) to ensure the temperature remained constant for each measurement. The seawater in the 300 l tank was kept air-saturated with a constant flow and water level was controlled by an overflow system. Temperature was regulated in the ambient tank using two 2 kW heaters (Austin and Cridland Pty. Ltd., NSW, Australia) and bubbling air ensured no temperature gradients occurred in the tank. The oxygen content in the chamber was measured with a luminescent dissolved oxygen optode (Hach LDO, HQ40d, Hach Company, USA) that logged dissolved oxygen recorded every 60 s. The oxygen optode was housed in a separate 6 ml chamber, which received seawater from the respirometry chamber via a recirculating peristaltic pump (PER-R, meacon Systems, TAS, Australia) to ensure proper mixing of seawater inside the 6 ml chamber and adequate flow passed the oxygen probe. Two submersible aquarium pumps (Quiet1one 1200, Aquasonic, Wauchope, NSW, Australia) were connected to the respirometers. One of these recirculated water inside the respirometer at a rate of 3.5 l min^{-1} to ensure proper mixing inside the chamber. The other pump was connected to a digital timer (DRT-1, Sentinel, China) which intermittently exchanged the water inside the respirometer at a rate of 3.5 l min^{-1} with seawater from the ambient tank for 10 min every 20 min, creating a 10 min closed and a 10 min flush cycle. This allowed a $\dot{M}O_2$ measurement every 20 min. To avoid interference from possible circadian behavioural patterns, lobster $\dot{M}O_2$ readings were always measured in constant light. Throughout the respirometry trial, the oxygen saturation was kept above 70% (Ikeda et al., 2000). Measurements of $\dot{M}O_2$ were taken over a period of 24 h once lobsters were treated (n= 4), equating to 72 $\dot{M}O_2$ measurements for each lobster. The time of treatment corresponded to 0 h. Background $\dot{M}O_2$ was measured in an empty chamber for 4 h

after each measurement and acted as controls. Oxygen consumption rates were expressed in mg O_2 g DM^{-1} h^{-1} after the subtraction of control measurements obtained from empty chambers.

Following the final measurement of $\dot{M}O_2$ for each animal the seawater in the respirometer was sampled for ammonia-N analysis (see below) and the total length (TL) and wet mass (WM) of each lobster was measured. The mass of each lobster was measured to the nearest 100 mg on a precision balance (Mettler SB24001 DeltaRange, Metler-Toledo, Switzerland). Values of wet mass were converted to dry mass (DM) for each animal based on moisture content of a triplicate sample of lobsters of a similar size. Dry mass was determined after rinsing lobsters in 0.5M ammonium formate to remove any salt and drying in an oven for 72 h at 60°C.

5.3.5 Metabolic responses

The mean of the lowest 10% of $\dot{M}O_2$ readings for each lobster was calculated to establish R_s (Herrmann and Enders, 2000; Enders and Herrmann, 2003; Ohlberger et al., 2007). Routine metabolic rate was defined as the average of $\dot{M}O_2$ readings for each lobster (Herrmann and Enders, 2000). The maximum metabolic rate of each reading was defined as the mean of the highest 10% of $\dot{M}O_2$ measurements for each lobster. The R_{active} was defined as the highest 10% of $\dot{M}O_2$ readings for each lobster in the Group E treatment. Metabolic states (R_s, R_r, and R_{max}) were calculated during the final 12 h of the 16 h acclimation period and for 24 h during the post-treatment period for all groups. The post-treatment metabolic responses associated with each treatment were compared to the mean R_r of all groups during the 12 h pre-treatment period. The duration of metabolic responses for each treatment was defined as the time (h) post-treatment $\dot{M}O_2$ remained above the mean pre-treatment R_r (plus standard error; SE) of all groups. The magnitude of the post-treatment metabolic response was

defined as the cumulative increase in $\dot{M}O_2$ (mg O_2 g DM^{-1} h^{-1}) above pre-treatment R_r for the duration of the metabolic response (Secor, 2009). The peak metabolic response for each treatment was defined as the highest $\dot{M}O_2$ reading for the 24 h post-treatment period (Secor, 2009). Factorial rise in $\dot{M}O_2$ was determined by the division of pre-treatment R_s from post-treatment R_{max}. The factorial rise in $\dot{M}O_2$ following exercise to exhaustion in Group E was used define FAS. The temporal distribution of the number of lobsters with the lowest 10% of hourly $\dot{M}O_2$ readings was defined as the percentage of individual lobsters that recorded R_s values (the lowest 10% of $\dot{M}O_2$) within each hourly block (i.e. 1-2 h, 2-3 h) for the 24 h post-treatment period.

5.3.6 Ammonia-N excretion rate

At the end of each respiratory trial (24 h after application of each treatment), the chamber closed cycle was extended to 20 min, during which time $\dot{M}O_2$ was simultaneously recorded and used to determine the O:N ratio. A 50 ml sample of seawater was then drawn from each chamber, sealed in 10% HCL acid-washed glass vials with a drop of 10% chloroform added to prevent bacterial growth and stored at -20°C prior to analysis. The ammonia-N concentration in these samples was determined using a salicylate-hypochlorite method following the procedure of Bower and Holm-Hansen (1980) and expressed in mg NH_4-N g DM^{-1} h^{-1} after the subtraction of control ammonia-N from the corresponding blank chamber.

Atomic O:N ratios were determined by the division of oxygen consumption (mg O_2 g DM^{-1} h^{-1}) and ammonia-N excretion rates (mg NH_4-N g DM^{-1} h^{-1}), using the atomic masses of O_2 (32.00) and N (14.01).

5.3.7 Data analysis

Oxygen consumption rates of lobsters and controls were determined using linear regression of the rate of decline in dissolved oxygen concentration for the final 8 min of every 10 min closed respirometer cycle. Only measurements with regression coefficients (R^2) above 0.95 were used to calculate $\dot{M}O_2$ of lobsters and background $\dot{M}O_2$. Residual plots were used to explore normality and homogeneity of data. Differences in mean $\dot{M}O_2$ and ammonia-N excretion rates were compared using one-way analysis of variance (ANOVA) and significant differences identified using Tukey's HSD tests for post-hoc multiple comparisons. Measurements of metabolic rates for acclimation and treatment periods were compared using a paired sample t-test. The level of significance for all analyses was determined at $P<0.05$. Data are presented as mean ± standard error (SE) unless stated otherwise. Statistical analyses were performed using SPSS version 16.0 (2007 SPSS Inc.).

5.4 Results

5.4.1 Oxygen consumption rate

Baseline $\dot{M}O_2$ (12 h pre-treatment period) was not significantly different between groups for R_r, R_s, and R_{max} (Table 5.1). Oxygen consumption rate increased following all treatments. All treatments reached their highest $\dot{M}O_2$ immediately after treatment and then gradually declined to pre-treatment R_r levels, apart from Group E lobsters which displayed a second increase in $\dot{M}O_2$ after approximately 4 h. There were no significant differences in post-treatment R_r, post-treatment R_s, post-treatment R_{max}, or post-treatment peak in $\dot{M}O_2$ amongst treatment groups (Table 5.1).

The duration of the post-treatment response in $\dot{M}O_2$ was significantly higher in Group E lobsters compared to Group A lobsters (Table 5.1). The duration of the post-treatment response was similar for all other treatments.

The magnitude of the post-treatment response in $\dot{M}O_2$ was significantly larger in Group E animals compared to Group A lobsters (Table 5.1). The magnitude of the post-treatment response in $\dot{M}O_2$ was similar between all other treatments groups.

The factorial rise in $\dot{M}O_2$ was not significantly different between treatments (Table 5.1). The lowest increase in $\dot{M}O_2$ above pre-treatment R_s was observed following handling (Group A) and the highest increase occurred following exercise to exhaustion (Group E).

The temporal distribution of the proportion of lobsters with the lowest 10% of hourly $\dot{M}O_2$ readings was reasonably equal over the 24 h measurement period for Group A, with the majority of the lowest readings (15%) occurring after 20 h (Fig. 5.2A). The first of the lowest 10% of $\dot{M}O_2$ readings were recorded 3 h post-treatment in Group A lobsters. The earliest of the lowest 10% of $\dot{M}O_2$ were recorded after 5 h in

Group B and the eight hour period, 5-12 h post-treatment, represented 82% of the proportion of lobsters with the lowest 10% of hourly $\dot{M}O_2$ measurements (Fig. 5.2B). A period of three hours, 9-11 h post-treatment, accounted for 45% of the proportion of lobsters with the lowest 10% of $\dot{M}O_2$ measurements in Group C, with the first of the lowest 10% of $\dot{M}O_2$ readings recorded after 7 h post-treatment (Fig. 5.2C). The temporal distribution of the proportion of lobsters with the lowest 10% of $\dot{M}O_2$ recorded for Group D lobsters was more equal over the 24 h measurement period, with the earliest of the lowest 10% of readings occurring 5 h post-treatment (Fig. 5.2D). The proportion of lobsters with the lowest 10% of $\dot{M}O_2$ readings was concentrated at the end of the measurement period in Group E lobsters, with the majority of the lowest readings (18%) occurring after 21 h (Fig. 5.2E). The first of the lowest 10% of $\dot{M}O_2$ readings in Group E was recorded 9 h post-treatment.

5.4.2 Ammonia-N excretion rate

The mean ammonia-N excretion rates of lobsters were not significantly different between treatment groups ($F= 1.523$, df 4, 19, $P= 0.246$, Fig. 5.3A). The mean atomic O:N ratio for lobsters was also not significantly different between groups ($F= 0.401$, df 4, 19, $P= 0.805$, Fig. 5.3B).

Table 5.1. Summary of oxygen consumption rate ($\dot{M}O_2$) parameters (mean ± SE) of routine metabolic rate (R_r), standard metabolic rate (R_s) and maximum metabolic rate (R_{max}) during the pre-treatment (12 h acclimation) period and 24 h post-treatment period and the metabolic responses of post-treatment duration, magnitude, peak, and factorial rise in $\dot{M}O_2$ for Group A, B, C, D, and E juvenile *Sagmariasus verreauxi*.

	Group A	**Group B**	**Group C**	**Group D**	**Group E**	**F 4, 19**	***P***
Pre-treatment							
Pre-treatment R_r (mg O_2 g DM^{-1} h^{-1})	0.291 ± 0.025	0.212 ± 0.023	0.222 ± 0.020	0.333 ± 0.061	0.231 ± 0.036	2.073	0.135
Pre-treatment R_s (mg O_2 g DM^{-1} h^{-1})	0.168 ± 0.015	0.145 ± 0.026	0.165 ± 0.014	0.229 ± 0.038	0.151 ± 0.019	1.941	0.156
Pre-treatment R_{max} (mg O_2 g DM^{-1} h^{-1})	0.491 ± 0.018	0.438 ± 0.056	0.436 ± 0.022	0.495 ± 0.062	0.413 ± 0.046	0.670	0.623
Post-treatment							
Post-treatment R_r (mg O_2 g DM^{-1} h^{-1})	0.259 ± 0.027	0.279 ± 0.057	0.305 ± 0.029	0.342 ± 0.039	0.356 ± 0.013	1.300	0.314
Post-treatment R_s (mg O_2 g DM^{-1} h^{-1})	0.178 ± 0.016	0.177 ± 0.040	0.210 ± 0.021	0.232 ± 0.020	0.200 ± 0.007	0.965	0.455
Post-treatment R_{max} (mg O_2 g DM^{-1} h^{-1})	0.437 ± 0.025	0.454 ± 0.084	0.503 ± 0.040	0.516 ± 0.050	0.536 ± 0.008	0.739	0.580
Duration (h)	2.50 ± 1.40^{a}	4.50 ± 1.77^{ab}	4.25 ± 0.87^{ab}	5.17 ± 2.21^{ab}	10.17 ± 0.70^{b}	3.705	0.027
Magnitude	0.294 ± 0.237^{a}	0.721 ± 0.356^{ab}	0.646 ± 0.169^{ab}	0.797 ± 0.409^{ab}	1.835 ± 0.128^{b}	4.242	0.017
Peak (mg O_2 g DM^{-1} h^{-1})	0.530 ± 0.010	0.499 ± 0.079	0.539 ± 0.029	0.560 ± 0.046	0.567 ± 0.016	0.376	0.822
Factorial rise	2.66 ± 0.24	3.12 ± 0.05	3.07 ± 0.20	2.43 ± 0.44	3.73 ± 0.48	2.343	0.102

F and *P* statistics refer to one-way ANOVA on the factor Group for the corresponding row. Letter superscripts indicate significant differences ($P<0.05$).

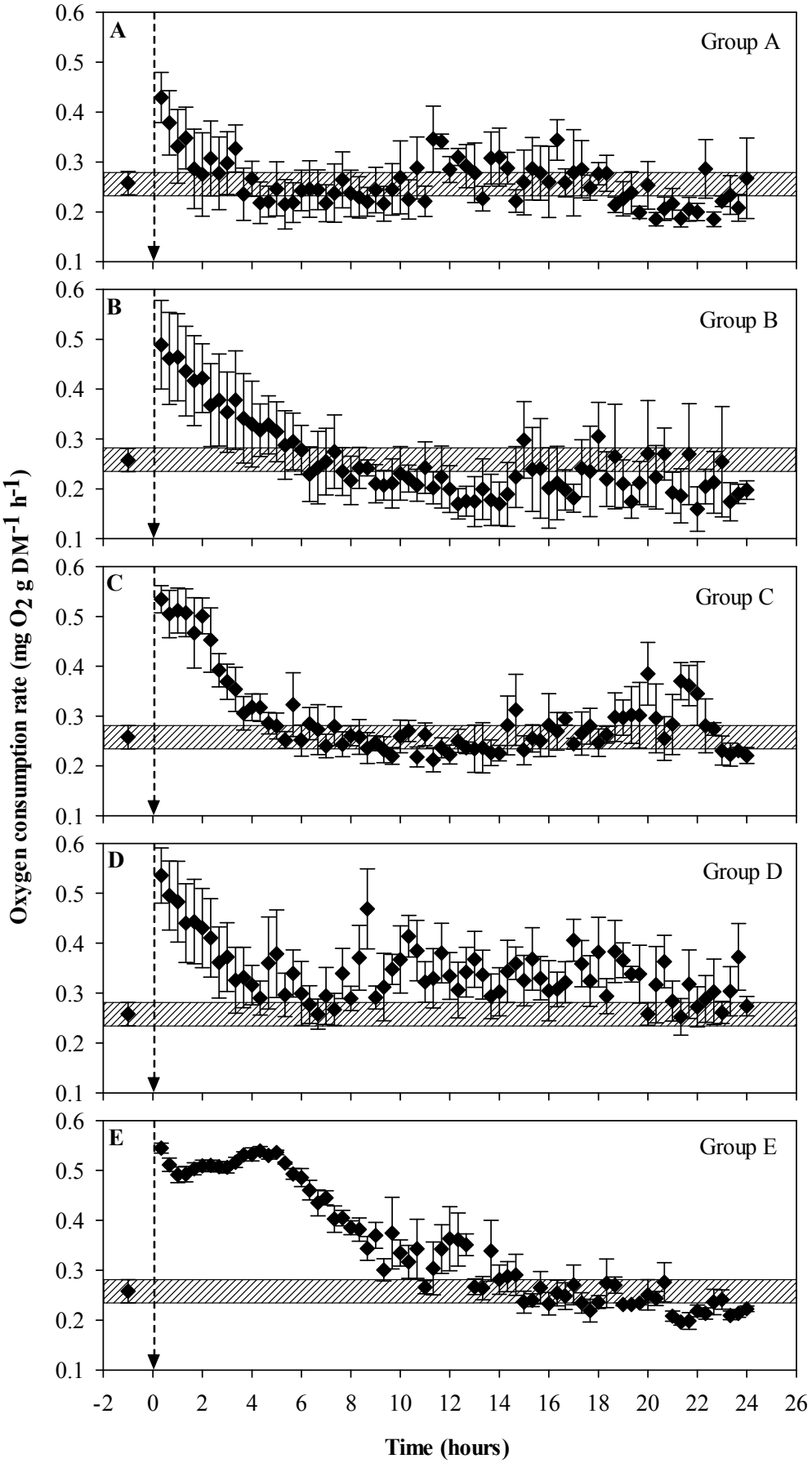
A
Group A
B
Group B
C
Group C
D
Group D
E
Group E
Oxygen consumption rate (mg O2 g DM-1 h-1)
Time (hours)
0.1
0.2
0.3
0.4
0.5
0.6
-2
0
2
4
6
8
10
12
14
16
18
20
22
24
26

Figure 5.1. Oxygen consumption rate ($\dot{M}O_2$) of juvenile *Sagmariasus verreauxi* for a post-treatment period of 24 h after: (A) handling (Group A); (B) anaesthesia with 2% 2-phenoxyethanol (Group B): (C) anaesthesia with 2% 2-phenoxyethanol and immediately force feeding 1% body mass (BM) seawater (Group C); (D) anaesthesia with 0.032% AQUI-S and immediately force feeding 1% body mass (BM) seawater (Group D) and; (E) swimming to exhaustion (Group E). The shaded area represents the mean pre-treatment R_r ± SE of all groups. Arrows indicate where lobsters were treated a time 0 h. Values are mean (± SE), n= 4.

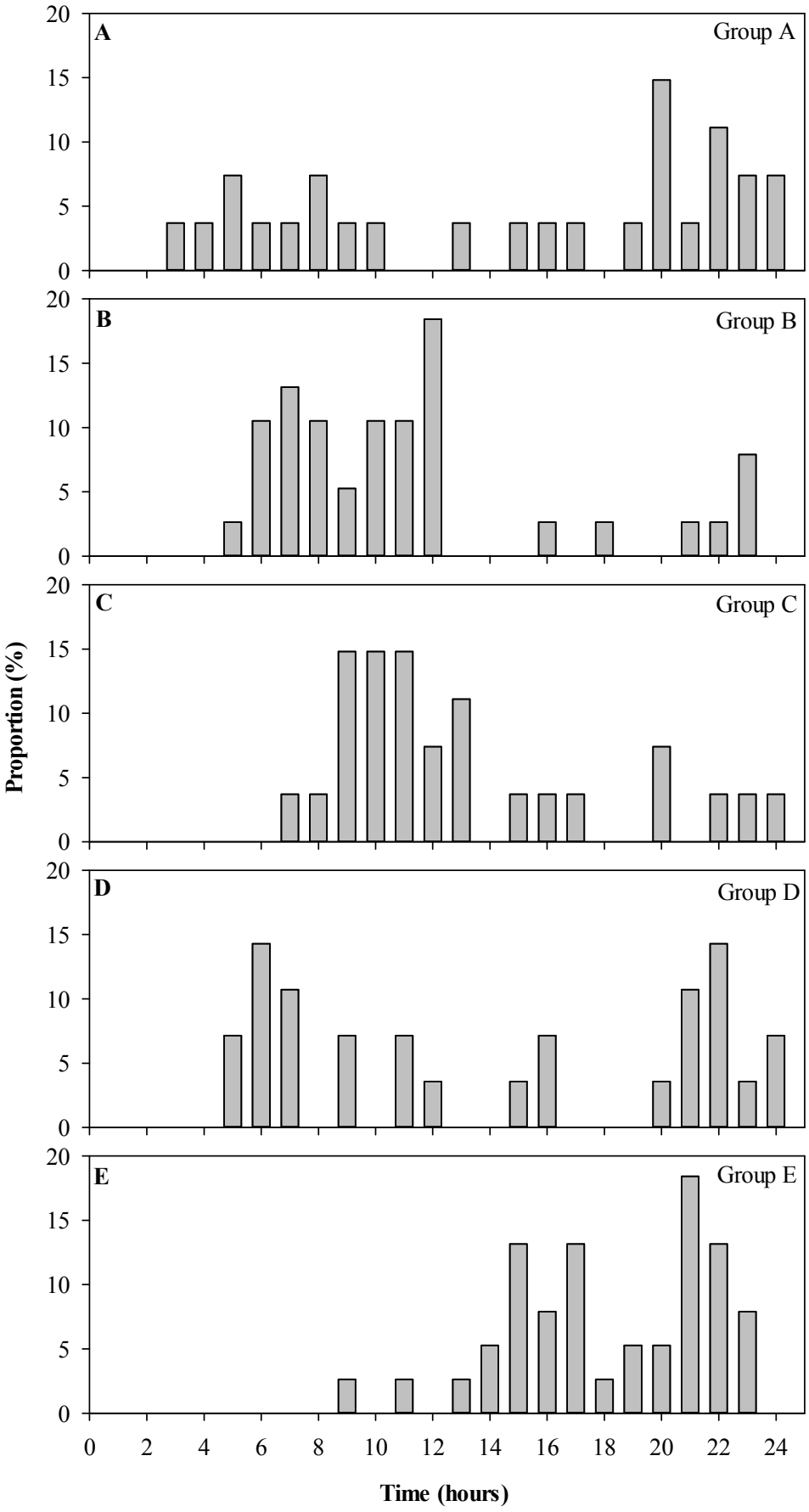
A
Group A
B
Group B
C
Group C
D
Group D
E
Group E
Proportion (%)
0
5
10
15
20
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)

Figure 5.2. Temporal distribution of the proportion (%) of juvenile *Sagmariasus verreauxi* with the lowest 10% oxygen consumption rate ($\dot{M}O_2$) readings in each hourly block for a post-treatment period of 24 h after: (A) handling (Group A); (B) anaesthesia with 2% 2-phenoxyethanol (Group B): (C) anaesthesia with 2% 2-phenoxyethanol and immediately force feeding 1% body mass (BM) seawater (Group C); (D) anaesthesia with 0.032% AQUI-S and immediately force feeding 1% body mass (BM) seawater (Group D) and; (E) swimming to exhaustion (Group E). n= 4.

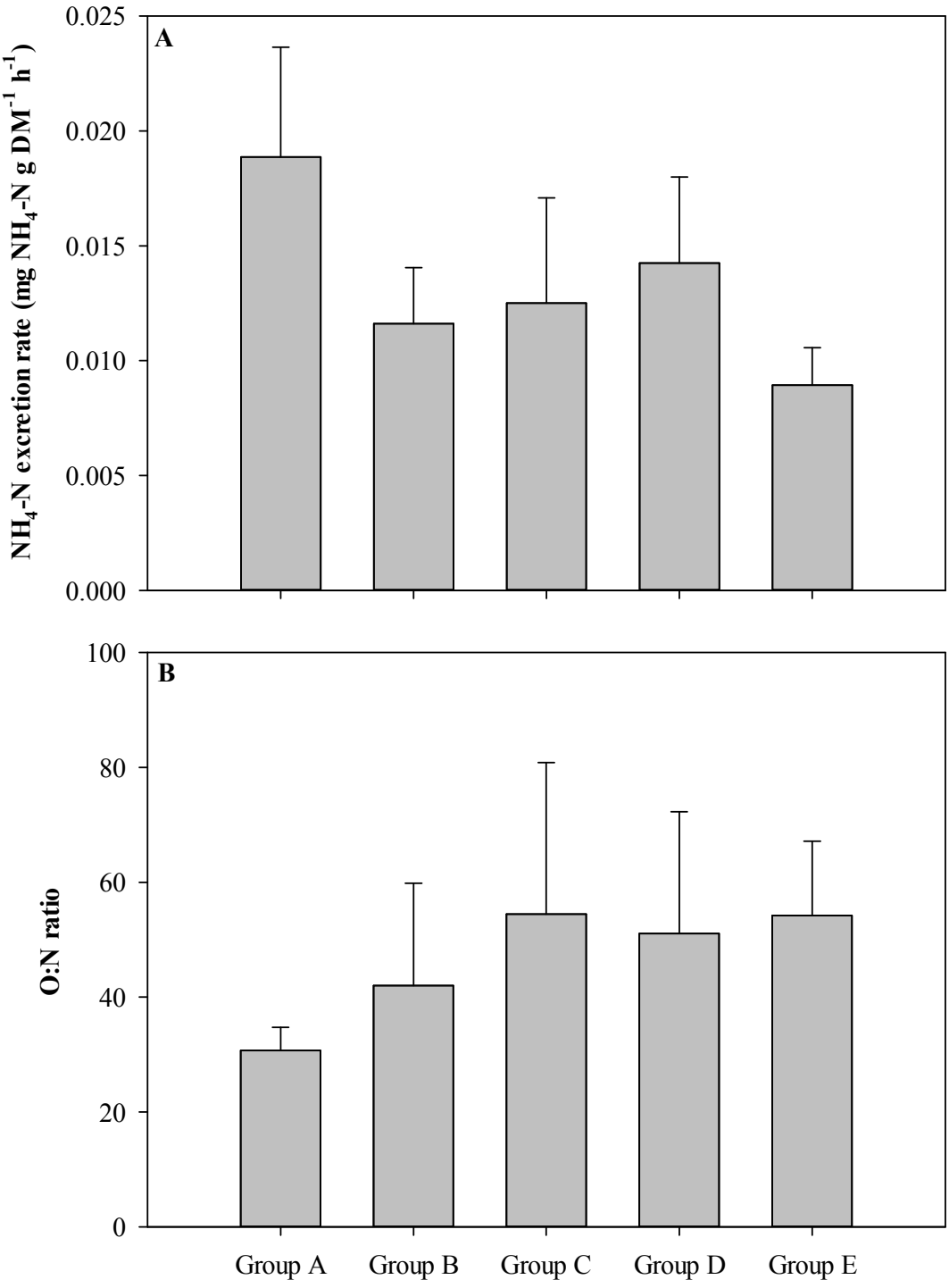

Figure 5.3. (A) Ammonia-N (**(**NH_4-N) excretion rate and; (B) atomic O:N ratio of juvenile *Sagmariasus verreauxi* 24 h after: handling (Group A); anaesthesia with 2% 2-phenoxyethanol (Group B): anaesthesia with 2% 2-phenoxyethanol and immediately force feeding 1% body mass (BM) seawater (Group C); anaesthesia with 0.032% AQUI-S and immediately force feeding 1% body mass (BM) seawater (Group D); and swimming to exhaustion (Group E). Values are mean (±SE), n= 4.

5.5 Discussion

5.5.1 Factorial aerobic scope

The present study demonstrated that an intensive chase protocol is an effective method for estimating FAS of juvenile *S. verreauxi* and exercising to exhaustion caused the largest increase in $\dot{M}O_2$. Following exercise to exhaustion, $\dot{M}O_2$ of *S. verreauxi* increased 3.7 ± 0.5 times pre-treatment R_s and reached peak levels of 0.567 ± 0.016 mg O_2 g DM^{-1} h^{-1} shortly after activity. An increase by 3.7 times for *S. verreauxi* is in the range of 2-6 typically reported for most aquatic and terrestrial crustaceans (McMahon et al., 1979; Wood and Randall, 1981; McMahon and Wilkens, 1983; Full and Herreid, 1984; Full et al., 1985; Houlihan et al., 1985; Wheatly et al., 1985; Herreid and Full, 1986; Hamilton and Houlihan, 1992) but is considerably lower than the highest values (8-12 times R_s) recorded for some terrestrial crabs (Full and Herreid, 1983; Full, 1987; Maitland, 1987). The higher FAS of terrestrial crabs may be necessary to facilitate the more active nature of these crab species compared to relatively inactive benthic spiny lobsters (Crear and Forteath, 2000). Many terrestrial crabs also have modified gills or accessory gas exchange organs (usually lungs) to assist in gas exchange (Morris, 2002). Alternatively, benthic lobsters are almost neutrally buoyant whereas some terrestrial crabs may have a higher FAS on land in order to deal with the energetic costs imposed on their bodies by gravity compared to when they are immersed in water (Power et al., 1991). Factorial aerobic scope of *S. verreauxi* is also generally well below that of many active fish species (up to 12 times R_s) (Brett, 1972). The lower FAS of *S. verreauxi* in this situation may be explained by the limitations of oxygen

diffusion over chitin on the gills (Taylor, 1982) and frequency of scaphognathite pumping limiting branchial water flow (McMahon et al., 1979).

5.5.2 Recovery period

Similar to the trend in all other treatments in the present study, $\dot{M}O_2$ was initially high and gradually decreased following activity. However, the aerobic response of *S. verreauxi* during recovery from exercising to exhaustion followed a slightly different pattern than the other treatments, with $\dot{M}O_2$ increasing again after approximately 4 h. During intensive exercise in lobsters, energy requirement exceeds aerobic capacity and, therefore, tail-flipping is fuelled anaerobically (England and Baldwin, 1983). Anaerobic tail-flipping is initially powered by the hydrolysis of phosphagen arginine phosphate (AP) and then once these pools are nearly depleted energy is supplied by anaerobic glycogenolysis, which is characterised by the accumulation of lactate and depletion of glycogen (England and Baldwin, 1983; Booth and McMahon, 1985; Head and Baldwin, 1986; Milligan et al., 1989; Morris and Adamczewska, 2002). Crustaceans presumably utilise anaerobic metabolism during intensive exercise to accelerate important phases of recovery such as the rate of resynthesis of AP, which would otherwise be restricted by oxygen diffusion across their large muscle fibres (Kinsey and Moerland, 2002; Boyle et al., 2003; Johnson et al., 2004; Kinsey et al., 2005). However, reliance on anaerobic metabolism places crustaceans further in oxygen debt and total recovery must be aerobic (Jimenez et al., 2008), which may explain why $\dot{M}O_2$ in juvenile *S. verreauxi* increased again after 4 h following exercise to exhaustion. This second increase in $\dot{M}O_2$ is consistent with other studies where $\dot{M}O_2$ during recovery exceeds the predicted aerobic oxygen deficit (Herreid, 1980). However, only a small proportion of metabolic recovery following swimming to exhaustion in crustaceans was found to be dedicated to AP resynthesis

(Jimenez et al., 2008), which suggests that other processes such as metabolism of anaerobic end products (such as lactate), restoring standard oxygen concentrations in body tissues, and accounting for energy costs associated with increased ventilation and haemolymph circulation may be additional reasons $\dot{M}O_2$ increased 4 h after exercise to exhaustion (Herreid, 1980).

Oxygen consumption rates following exercise to exhaustion remained above pre-treatment R_r for a period of 10.2 h in the present study. This is comparable to recovery periods from previous studies on induced activity of other crustaceans (McMahon et al., 1979) and air-exposure of *Panulirus cygnus* (Crear and Forteath, 2001a; 2001b) where $\dot{M}O_2$ increased by a similar magnitude and returned to normal levels within approximately 8 h. Crear and Forteath (2001b) suggested the duration and effectiveness of recovery processes resulting from activity should be adequately efficient for the animal to deal with additional periods of stress. Likewise, recovery must be rapid in muscles used for escape responses to allow the animal to continue to escape from predators (Ellington, 1983).

Comparisons of R_s between species and treatment groups of animals are common. However, the data used in these comparisons may not be accurately comparable because evaluation of R_s may be biased by trial length and calculation interval (Hayes et al., 1992). Variables such as the time elapsed from placing animals in respirometers and recording $\dot{M}O_2$ measurements is often not compensated for and may account for significant variations in measurements between species (Kenagy and Vleck, 1982). Temporal distribution of the lowest $\dot{M}O_2$ measurements for different treatments showed they were more abundant toward the end of the 24 h measurement period, although numerous low values were recorded earlier following anaesthesia with 2-phenoxyeyhanol. Even though $\dot{M}O_2$ returned to pre-treatment levels 2.5 h after

handling, the majority of the lowest readings occurred after 20 h. This time period suggests that juvenile *S. verreauxi* of this size may require a 20 h acclimation period following handling before accurate estimations of R_s can be determined. Theoretically, R_s is the lowest rate of energy consumption required for maintaining life, but due to methodological aspects and the possibility of producing erroneous readings, it is generally preferred to include more than a single value when estimating R_s. This is one advantage of intermittent flow-through respirometry, which not only eliminates the problems caused by activity but also allows repeated measurements of $\dot{M}O_2$ for short time intervals over long periods (Steffensen, 1989).

5.5.3 Stress

In situations where the original stressor is removed, physiological responses resulting from stress are usually reversible (Taylor et al., 1997). For example, although most of the treatment groups in this study simulated situations that lobsters would not encounter in their natural environment, the primary physiological changes associated with each treatment may be fully reversed on re-immersion in seawater. This recovery is possible because the necessary compensatory mechanisms are similar to those already evolved to deal with normal stressors encountered in the wild such as intensive activity (Taylor et al., 1997). Observations of increased $\dot{M}O_2$ in lobsters are therefore more an indication that the lobster is temporarily challenged. Furthermore, individuals not only differ in their level of metabolic response to a specific stress stimulus, but they also differ in the time taken to restore normal behaviour and physiological processes following stress (Øverli et al., 2007). These differences are an important consideration for evaluating the time course of stressors when assessing various husbandry procedures.

This study clearly demonstrated that $\dot{M}O_2$ of juvenile *S. verreauxi* was elevated for more than 2 h after handling. The primary reason handling affects metabolic rates of lobsters, or any other crustacean, is by increasing their activity and energy expenditure during handling (Paterson, 1993). Animals also become more active during the initial period of a respirometry trial because of handling and the unfamiliar confines of a respirometry chamber (Careau et al., 2008). In lobsters, this activity is usually associated with exploring the chamber through "walking" and movement of limbs such as antennae, and sometimes even tail-flipping as an escape response to being handled. Increased $\dot{M}O_2$ due to transfer stress are commonly observed in respirometry studies (Cech, 1990) and the period required for metabolic rate to become stable is referred to as the adaption phase (Herrmann and Enders, 2000). Respirometry trials should, therefore, exclude measurements during the adaption phase in order to accurately determine metabolism, which can vary greatly between species and individuals (Herrmann and Enders, 2000; Steffensen, 2002). For this reason, it is also important to determine the required adaption phase for a specific species prior to making any comparisons. For example, Fitzgibbon (2010) demonstrated that metabolic rate of *S. verreauxi* pueruli was elevated for over 2 h following transfer to respirometers. However, Lemmens (1994) determined metabolic rates of *P. cygnus* pueruli within 30 min of transfer, which may represent elevated levels of metabolism.

5.5.4 Anaesthesia

Chemical anaesthetics are commonly used to reduce metabolic stress in fish for a range of procedures involving handling (Collins, 1990). However, little is known about the effects of most of these anaesthetics on crustaceans. Of the different anaesthetics tested for use in crustaceans, tricane methane sulfonate (MS222),

quinaldine, and 2-phenoxyethanol are not effective in prawns (Coyle et al., 2004; 2005). Conversely, clove oil, AQUI-S (Coyle et al., 2005), and cold anaesthesia using chilled sawdust (Salin, 2005) were found to be suitable anaesthetics for survival of *Macrobrachium rosenbergii*. However, these anaesthetics have not been tested on spiny lobsters. 2-phenoxyethanol is an oily liquid that is a constituent of photocopy fluid. The mode of action has not been cited, but is thought to involve the expansion of neuronal cell membranes. Withdrawal times have also not been established for 2-phenoxyethanol and is, therefore, not registered or approved for use in fish destined for human consumption (Burka et al., 1997), but is used in research (Coyle et al., 2005). AQUI-S is a relatively new anaesthetic comprising the active ingredient isoeugenol, which is a component of clove oils, and is approved for use in aquaculture in Australia, Chile, and New Zealand with no withholding period (Small, 2004). The mode of action has not been cited, but functional activity of the cardiovascular system in fish is maintained for extended periods (Burka et al., 1997).

To our knowledge the effects of anaesthesia on $\dot{M}O_2$ of crustaceans during recovery have not been studied previously. Previous studies on the effects of anaesthesia on $\dot{M}O_2$ of crustaceans and fish have been restricted to measuring $\dot{M}O_2$ during anaesthesia and were not assessed during recovery. These previous studies found a reduction in $\dot{M}O_2$ of adult *M. rosenbergii* anaesthetised with eugenol (Saydmohammed and Pal, 2009) and also in various fish species during sedation with numerous other anaesthetics (Teo and Chen, 1993; Wedemeyer, 1996; Kaiser and Vine, 1998; Hoskonen and Pirhonen, 2004). In the present study, the magnitude and duration of the metabolic response was significantly higher following exercise to exhaustion compared to handling. Techniques used to reduce activity, such as anaesthesia, should therefore minimise metabolic stress and maintain the health of

lobsters (Taylor et al., 1997). However, even though the use of anaesthetics in the current experiment (both 2-phenoxyethanol and AQUI-S) reduced activity and $\dot{M}O_2$ compared to R_{active}, they did not appear to reduce $\dot{M}O_2$ in lobsters when compared to the handling treatment. Oxygen consumption rates returned to pre-treatment R_r levels in lobsters 2.5 h after handling, but remained above pre-treatment R_r levels for 4.3 h and 5.2 h following anaesthesia with 2-phenoxyethanol and AQUI-S, respectively. Maximum metabolic rate of lobsters anaesthetised with AQUI-S and 2-phenoxyethanol was also slightly higher than R_{max} following handling and the peak $\dot{M}O_2$ of lobsters anaesthetised with AQUI-S approached that of the peak following exercise to exhaustion. This suggests that although anaesthetics reduced activity of lobsters, they do not eliminate the effect of handling stress and actually cause an increase in $\dot{M}O_2$. In the case of AQUI-S, a large proportion of aerobic scope is utilised.

5.5.5 Force feeding

Force feeding diets may be a method for ensuring all dietary components are ingested by lobsters and allow more accurate comparisons of SDA responses. Research on force feeding diets is extremely limited, with the majority of previous studies restricted to catheterising or anaesthetising fish and forcing food directly into their stomachs to reduce the metabolic cost of activity during feeding (Thorarensen and Farrell, 2006; Dupont-Prinet et al., 2010). However, even after anaesthesia, these force feeding protocols indicated that handling increased metabolic rates for several hours, requiring these treatments to correct for any contribution of handling to the SDA response (Dupont-Prinet et al., 2010). Lobsters are also very difficult to force feed and will rapidly reject any tubes inserted into their mouths. Therefore in the present study, anaesthetics were adopted to enable force feeding of lobsters and

reduce the effect of handling and activity on metabolic responses. Anaesthesia allowed force feeding without lobsters rejecting the tube or saline solution, however it resulted in a dramatic increase in $\dot{M}O_2$ of 2.4 and 3.0 times pre-treatment R_s for AQUI-S and 2-phenoxyethanol, respectively. This increase is only slightly below the 3.7 times increase in $\dot{M}O_2$ following exercise to exhaustion, suggesting that anaesthesia causes a considerable physiological stress response in juvenile lobsters and uses a large proportion of the aerobic scope. The increase in $\dot{M}O_2$ following anaesthesia also remained above pre-treatment R_r for up to 5.2 h (AQUI-S). However, SDA responses have been reported to peak at 10-13 h in various juvenile spiny lobster species (Crear and Forteath, 2000; Radford et al., 2008) and last for up to 46 h (Crear and Forteath, 2001a). Therefore, given the benefit of force feeding ensuring all dietary components are ingested, this may be a suitable protocol for force feeding lobsters, provided the metabolic responses to anaesthesia are adjusted for.

5.5.6 Energy utilisation

In addition to decreasing activity, anaesthetics may also reduce ammonia production, which is particularly beneficial during fish transportation (Wedemeyer, 1996; Ross and Ross, 1999). Conversely, a number of laboratory studies have shown ammonia excretion is elevated for 1-2 h following handling stress (Spaargaren et al., 1982; Regnault, 1984; Willason and Johnson, 1986; Hunter and Uglow, 1993), which is consistent with the general trend of ammonia-N excretion rates in the present study. However, ammonia-N excretion rates in the present study were calculated 24 h post-treatment and may not provide an accurate representation of the immediate stress response each treatment incurred on ammonia-N excretion. The $\dot{M}O_2$ of lobsters was also simultaneously calculated 24 h post-treatment to determine atomic O:N ratios. Atomic O:N ratios are useful for evaluating nutrients utilised by ammoniotelic

animals and can provide information on changes in energy substrate utilisation under various conditions (Corner and Cowey, 1968; Carter and Brafield, 1991). However, the requirement to simultaneously measure ammonia-N excretion and $\dot{M}O_2$ is limited due to the individual variations in metabolism that may be a consequence of using a single measurement. An O:N ratio of 9.2 indicates only protein is being metabolised (if under aerobic conditions) where as a ratio of above 18.5 indicates more than half the oxygen is being used to metabolise non-protein substrates (Brafield, 1985). Protein dominated metabolism (low O:N ratio) in crustaceans is generally an indication of stressful conditions (Pillai and Diwan, 2002). However, atomic O:N ratios for all groups in the present study were higher than 18.5 demonstrating lipid based metabolism which, in accordance with $\dot{M}O_2$ and ammonia-N excretion measurements, indicates lobsters were fully recovered 24 h post-treatment in all groups.

5.5.7 Conclusion

The results of the present study demonstrated that *S. verreauxi* juveniles have a narrow aerobic scope and, therefore, limited ability to respond to stressors. An increase in $\dot{M}O_2$ induced by force feeding and anaesthesia uses a large proportion of energy within the aerobic scope that could otherwise be utilised for other physiological functions, such as growth and development. Handling did not cause a large increase in $\dot{M}O_2$ and lobsters recovered rapidly, suggesting they quickly gain full capacity to perform typical behaviours and avoid predators following handling. Exercise to exhaustion, however, resulted in a large increase in $\dot{M}O_2$ and extended recovery periods, which may reduce growth and increase disease susceptibility and the risk of predation. Therefore, it is recommended that aquaculture handling procedures make precautions not to exhaust lobsters and are carried out without

anaesthesia to facilitate rapid recovery of lobsters. Finally, anaesthesia allowed force feeding of lobsters and may provide an improved method for physiological trials compared to previous SDA protocols if metabolic responses to anaesthesia are accounted for.

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THERMAL TOLERANCE OF JUVENILE SPINY LOBSTERS *Sagmariasus verreauxi* AND THE EFFECT OF BODY MASS

Chapter VI

6.1 Abstract

Studies of thermal tolerance in marine organisms are crucial in understanding climate effects on their natural distribution; however, thermal tolerance has rarely been investigated in different sized animals of the same species. A mismatch between oxygen demand and the limited capacity of oxygen supply to tissues is hypothesised to be the first mechanism controlling survival at the thermal tolerance limits of marine organisms. Therefore, thermal windows; defined by low and high *pejus* (T_p) and critical temperatures (T_c); of large (410-659 g) and small (28-44 g) juvenile *Sagmariasus verreauxi* were examined in order to better understand and predict their future range expansion. Temperature thresholds were determined primarily from the combined shift in trends of standard metabolic rate (R_s) and haemolymph O_2 concentration. Temperature-dependent ammonia-N excretion rate, O:N ratio, and haemolymph pH, clotting time, glucose, lactate, ammonia, and protein concentrations were also examined. Sudden changes in haemolymph O_2 concentration below 12°C and above 26°C in large lobsters and below 10°C and above 24°C in small lobsters indicated the existence of low and high T_p thresholds, respectively. Maximum R_s reached at high temperatures indicated the presence of the upper T_c threshold at 30°C for both lobster size classes. Temperature coefficients (Q_{10}) supported the presence of T_p thresholds with a value of 2.7 for both lobster size classes within the thermal optimum window, but were very high below the low T_p threshold. Atomic O:N ratios revealed that juvenile lobsters utilise more lipid as an energy substrate, but shifted toward protein catabolism above T_p thresholds. Exposure to temperatures beyond the thermal tolerance window (>30°C) was characterised by the onset of anaerobic metabolism, which was detected by haemolymph acidosis (due to an increase in

lactate), hyperglycaemia, some accumulation of protein, but not of ammonia. The present study is the first to demonstrate evidence of oxygen related thermal tolerance in juvenile spiny lobsters. Both size classes of juvenile *S. verreauxi* have a wide thermal tolerance window, suggesting they have the ability to expand their distribution if confronted with temperatures approaching T_c and T_p in the wild. This expanded distribution may have a large impact on benthic community structures and dynamics, which is an important consideration for future research.

6.2 Introduction

A number of factors have been identified as affecting the natural distribution of marine organisms, ranging from competition, predation, habitat patchiness, and habitat area (Rohde, 1992; Rosenzweig, 1992; Roy et al., 1994) to climatic variability, differential extinction, and biogeographic boundaries (Stevens, 1989; Blackburn and Roy et al., 1994; Smith et al., 1994; Brown, 1995; Gaston, 1996; Gaston et al., 1998). However, from a physiological viewpoint it has recently been shown that the distribution of marine organisms is also strongly related to oxygen supply (Verberk et al., 2011) and temperature (Anger, 2001; Pörtner, 2001). In the latter sense, aquatic organisms are adapted to and depend on the maintenance of the thermal window (temperature range within the thermal tolerance limits of an organism) within their natural environment (Pörtner, 2001). Previous studies have mainly focussed on the thermal tolerance of adult animals (Frederich and Pörtner, 2000; Stillman, 2002; Lee, 2003; Fangue et al., 2006; Compton et al., 2007; Wittmann et al., 2008) and have rarely investigated different sized animals of the same species (Frederich and Pörtner, 2000; Melzner et al., 2006; Pörtner and Knust, 2007; Wittmann et al., 2008). However, thermal tolerance has recently been shown to vary between life stages (Anger et al., 2003; Parker et al., 2009), which can have important consequences on population dynamics and biogeographic limits of many aquatic animals (Storch et al., 2011). In spiny lobsters, thermal tolerance of juveniles is particularly important considering the potential for this parameter to affect their distribution given the variations in temperature that *Sagmariasus verreauxi* experience across a wide distribution (Montgomery and Craig, 2005) and in view of likely climate change events (Johnson et al., 2011).

Rising seawater temperatures associated with climate change has already been shown to affect the recruitment and distribution of *S. verreauxi* and other spiny lobster species (Johnson et al., 2011). *S. verreauxi* is classified as a warm temperate species (Jensen et al., 2011) and occurs in shallow waters of the South Pacific Ocean and Tasman Sea off the coast of Australia from Tweed Heads (28°S) to Tasmania (42°S) and also in New Zealand, mainly in the North Island (approximately 35°S) (Montgomery and Craig, 2005). Mean ambient habitat temperatures are between 11°C and 27°C for this distribution (Condie and Dunn, 2006), suggesting a large thermal tolerance window for *S. verreauxi*. However, this region is one of the fastest warming in the southern hemisphere, and considered a global hotspot (Johnson et al., 2011). Extension of the East Australian Current (EAC) further south has already expanded the distribution of *S. verreauxi* southward to Tasmania (Johnson et al., 2011) and effects of this wider distribution on community structure and dynamics and on local economies are unclear (Poloczanska et al., 2007). These questions are important given the diversity of benthic community structures and the high economic value of fisheries in south east Australia (Johnson et al., 2011), and in particular, the southern rock lobster fishery in Tasmanian. By determining the thermal tolerance of *S. verreauxi* we may better understand and predict their future distribution range and enable ecologists to consider possible effects of this expansion on benthic communities.

Given the effects of temperature on oxygen supply and demand, it is logical that many thermal responses originate from an organisms oxygen demand exceeding supply (Verberk et al., 2011). Hence, a mismatch between oxygen demand and the limited capacity of ventilatory and circulatory systems to supply oxygen to tissues is postulated to be the first mechanism controlling survival at the thermal tolerance

limits of marine organisms (Frederich and Pörtner, 2000; Mark et al., 2002; Pörtner and Knust, 2007). Thermal limitation is firstly effected at high hierarchical levels of organisation, the entire animal and its oxygen delivery system and then at lower cellular and molecular levels (Mark et al., 2002; Pörtner et al., 2005). There are two major thermal tolerance thresholds, which have been defined based on clear changes in oxygen supply by ventilatory and circulatory systems (Pörtner, 2001; Mark et al., 2002). The narrower of these thresholds is referred to as the lower and upper *pejus* temperatures (T_p; *pejus* meaning getting worse) and are characterised by declining levels of oxygen in the haemolymph and other body fluids (Storch et al., 2009) and the resulting decrease in aerobic scope and normal functioning of the organism (Frederich and Pörtner, 2000; Peck et al., 2004; Wang and Overgaard, 2007). Critical functions such as growth and activity become limited beyond T_p thresholds and the capacity of the ventilatory and circulatory systems are reduced progressively, resulting in a mismatch between oxygen supply and demand (Frederich and Pörtner, 2000; Peck et al., 2004; Pörtner and Knust, 2007). This theory explains how inadequate oxygen supply at either side of T_p thresholds determines the thermal window in animals, with the optimum close to the high T_p (Pörtner, 2010). If further cooling or warming occurs the second thermal tolerance threshold is reached, which is defined as the lower and upper critical temperature (T_c). The onset of anaerobic metabolism is a consequence of marine organisms reaching T_c (Frederich and Pörtner, 2000) and prolonged exposure to temperatures beyond T_c will inevitably result in death unless thermal acclimation occurs (Zielinski and Pörtner, 1996; Sommer et al., 1997).

There is little information about the physiological processes that are impaired at T_p and T_c thresholds which may be crucial in defining the thermal tolerance limits

of an organism. For invertebrates, low and high T_c thresholds are often defined from the relationship between standard metabolic rate (R_s) and temperature (Storch et al., 2011). Within this thermal tolerance window R_s typically increases exponentially with temperature (Pörtner et al., 2005; Wittmann et al., 2008). Beyond T_c thresholds, oxygen deficiency and physiological disturbances cause metabolic depression, which is characterised by a decrease in R_s (Melzner et al., 2006). Also, because haemolymph O_2 levels depend on oxygen consumption and are controlled by ventilation, shifts in oxygen consumption rate ($\dot{M}O_2$) and haemolymph O_2 levels would likely indicate thermal tolerance thresholds according to the concept of oxygen and capacity limited thermal tolerance (Walther et al., 2009). In crustaceans, exposure to temperatures beyond their thermal tolerance range is also generally identified by internal hypoxia (deFur et al., 1988; Varley and Greenaway, 1992), a distinct respiratory and metabolic acidosis due to an increase in haemolymph CO_2 and lactate (Truchot, 1975; Taylor and Wheatley, 1981), hyperglycaemia (Telford, 1968; Santos and Keller, 1993b) and an accumulation of metabolic by-products such as ammonia (Schmitt and Uglow, 1997b). However, most physiological studies on crustaceans have been limited to temperatures within the animals' thermal optimum window (between lower and upper T_p). There is, therefore, limited information on parameters in crustaceans that become compromised at the extremes of their thermal tolerance range. Physiological studies can assist in explaining the distribution of marine organisms and recognise mechanisms involved in their thermal sensitivity, which may become useful in predicting effects of climate change (Pörtner and Knust, 2007).

The overall objective of the present study was to determine whether there is a mismatch between oxygen demand and the capacity to supply oxygen to tissues in

two size classes (28-44 g and 410-659 g) of *S. verreauxi* juveniles. The specific aim was to determine the thermal optimum and tolerance of two size classes of *S. verreauxi* juveniles and the critical temperature at which oxygen supply to tissues becomes limited and lobsters switch from aerobic to anaerobic metabolism by integrating measurements at the whole animal ($\dot{M}O_2$ and body mass) and physiological (haemolymph O_2, pH, clotting time, lactate, glucose, ammonia, and protein concentrations) levels. Thermal tolerance thresholds were examined from acute temperature changes in two different size classes of *S. verreauxi* juveniles in order to ascertain differences in thermal windows between lobster sizes and better understand and predict their future range expansion. To my knowledge the present study is the first to determine how temperature might affect the physiology of *S. verreauxi* juveniles outside their thermal optimum window and how the thermal tolerance of these lobsters might identify potential climate change effects on the species' distribution.

6.3 Materials and methods

6.3.1 Experimental animals

Juvenile *S. verreauxi* [105-118 mm total length (TL); 28.2-44.0 g wet mass (WM) (small lobsters) and 250-350 mm TL; 410.4-658.5 g WM (large lobsters)] were reared from hatch at the Institute for Marine and Antarctic Studies (IMAS), Taroona, Hobart. Stock juveniles were held in captivity in a 4,000 l fibreglass tank (large lobsters) or 40 l tanks (small lobsters) from May to June under a regime of ambient photoperiod and 20°C water temperature and fed a combination of fresh whole blue mussels (*Mytilus edulis*) and commercial prawn pellet (Higashimaru, Vital No. 12, http://www.k-higashimaru.co.jp/) daily.

Lobsters were randomly distributed into two groups (Group A and Group B). Group A animals were progressively cooled from 20°C to experimental temperatures, and Group B animals were warmed from 20°C to experimental temperatures (see section 6.3.3) at a rate of 2°C h^{-1}. Experimental temperatures were 18°C, 16°C, 14°C, 12°C, 10°C, 8°C, 6°C, and 4°C for Group A animals and 22°C, 24°C, 26°C, 28°C, 30°C, 32°C, 34°C, and 36°C for Group B animals, with 20°C acting as the control temperature. Lobsters were allowed to acclimate to their final temperature for 4 h, which is similar to the acclimation period used previously to examine thermal tolerance in crustacean larvae by Storch et al. (2009). After 4 h acclimation $\dot{M}O_2$, ammonia-N excretion rate, and haemolymph lactate, pH, glucose, O_2 concentration, total protein concentration, ammonia concentration, and clotting time were measured. All haemolymph samples (800-1000 µl) were extracted from the pericardial cavity using either an 18 G x 3½" (1.20 x 90 mm) spinal needle (for large lobsters) or 21 G x

1½" (0.80 x 38 mm) needle (for small lobsters) pre-chilled at -20°C. Single lobsters were sampled at each temperature, with eight temperatures (plus control temperature of 20°C) for each group of animals (Group A and Group B animals) at two different lobster size classes (see section 6.3.8 for statistical model).

6.3.2 Experimental system

Juvenile lobsters were held individually in 40 l rectangular tanks under a regime of ambient photoperiod and 20°C water temperature with constant water flow. Tanks were covered with nets to prevent animals escaping. Artificial hides constructed from oyster mesh (5 mm mesh size) were placed into tanks to provide shelter. Lobsters were observed for moulting and then held until they reached the halfway point of their moult stage, as determined by the mean of their previous moult increments. Lobsters were not fed and left undisturbed for 72 h to ensure that all individuals were in the same post-prandial state before any measurements.

6.3.3 Oxygen consumption rate

The lobsters were placed into respirometers and allowed to acclimate for 16 h at the control temperature (20°C). To avoid interference from possible circadian behavioural patterns, $\dot{M}O_2$ of lobsters was always measured in constant light. Throughout the respirometry trial, the oxygen saturation was kept above 70% (Ikeda et al., 2000). Oxygen consumption rate was measured over a period of 4 h at the experimental temperature and the mean of the lowest 10% of $\dot{M}O_2$ measurements for each animal was used to establish R_s (Herrmann and Enders, 2000; Enders and Herrmann 2003; Ohlberger et al., 2007). Background $\dot{M}O_2$ was measured in a blank chamber (without an animal) at the same treatment temperature for 4 h after each measurement.

Oxygen consumption rates were measured in an automated intermittent flow-through respirometer system which consisted of either a 0.95 l (for small lobsters) or 10.4 l (for large lobsters) respirometry chamber. Oyster mesh (5 mm mesh size) was fitted to the lower half of the respirometry chambers so that lobsters had traction on an otherwise smooth surface and could remain quiescent (Dall, 1986). The chambers were immersed in a 300 l ambient tank (temperature bath) to ensure the temperature remained at the desired level for each measurement. Seawater was heated in two steps prior to entering this ambient tank in order to achieve the desired temperatures. Firstly, water was temperature controlled in a 1,800 l tank using an industrial heat/chill unit (Carrier, C010PHH7AA, Australia), which supplied seawater to two 300 l sumps. Secondly, the temperature was further regulated in these two sumps using a 2.4 kW heater in each sump (Istra Elements and Engineering Pty. Ltd., Caringbah, Australia). Seawater was then pumped into the 300 l ambient tank using submersible aquarium pumps (Quiet1one 3000, Aquasonic, Wauchope, NSW, Australia) where temperature was regulated using two 2 kW heaters (Austin and Cridland Pty. Ltd., NSW, Australia) for warm temperatures and two chiller units (Ratek Instruments Pty. Ltd., Victoria, Australia) for cold temperature treatments. The seawater in the 300 l ambient tank was kept air-saturated with a constant flow of water and bubbling air ensured no temperature gradients occurred in the tank. The oxygen content in the chamber was measured with a luminescent dissolved oxygen optode (Hach LDO, HQ40d, Hach Company, USA) that logged dissolved oxygen recorded every 60 s. The oxygen optode was housed in a separate 6 ml chamber, which received seawater from the respirometer via a recirculating peristaltic pump (PER-R, meacon Systems, TAS, Australia) at a rate of 0.3 ml s^{-1}. Two submersible aquarium pumps (Quiet1one 1200, Aquasonic, Wauchope, NSW, Australia) were

connected to each respirometer. One of these recirculated water inside the respirometer at a rate of 2 l min^{-1} (0.95 l chambers) or 5 l min^{-1} (10.4 l chambers) to ensure proper mixing inside the chamber. The other pump was connected to a digital timer (DRT-1, Sentinel, China) which intermittently exchanged the water inside the respirometer at a rate of 2 l min^{-1} (0.95 l chambers) or 5 l min^{-1} (10.4 l chambers) with seawater from the ambient tank for 10 min every 20 min, creating a 10 min closed and a 10 min flush cycle. This allowed a $\dot{M}O_2$ measurement every 20 min. Oxygen consumption rate was expressed in mg O_2 g DM^{-1} h^{-1} after the subtraction of control measurements obtained from blank chambers.

Following the final $\dot{M}O_2$ measurement of a lobster at each temperature treatment the seawater in the respirometer was sampled for ammonia-N analysis (see below) and the TL and WM of each lobster was measured. The mass of each lobster was measured to the nearest 100 mg on a precision balance (Mettler SB24001 DeltaRange, Metler-Toledo, Switzerland). Values of WM were converted to dry mass (DM) for each animal based on the average moisture content of a triplicate sample of similar sized lobsters. Dry mass was determined after rinsing lobsters in 0.5M ammonium formate to remove any salt and drying in an oven for 72 h at 60°C.

6.3.4 Ammonia-N excretion rate

At the end of each respirometry trial, the chamber closed cycle was extended to 20 min, during which time $\dot{M}O_2$ was simultaneously recorded and used to determine the O:N ratio. A 50 ml sample of seawater was then drawn from each chamber, sealed in 10% HCL acid-washed glass vials with a drop of 10% chloroform added to prevent bacterial growth and stored at -20°C prior to analysis. The ammonia-N concentration in these samples was determined using a salicylate-

hypochlorite method following the procedure of Bower and Holm-Hansen (1980) and expressed in mg NH_4-N g DM^{-1} h^{-1}.

Atomic O:N ratios were determined by the division of oxygen consumption (mg O_2 g DM^{-1} h^{-1}) and ammonia-N excretion rates (mg NH_4-N g DM^{-1} h^{-1}), using the atomic masses of O_2 (32.00) and N (14.01).

6.3.5 Haemolymph O_2 and pH

A 200 µl sample of pure haemolymph was extracted from the pericardial cavity of each lobster (see section 6.3.1) immediately following $\dot{M}O_2$ measurements and straightaway transferred to an eppendorf tube stored on ice. The haemolymph sample was then drawn from the eppendorf tube using a 1 ml syringe and injected past an oxygen minisensor probe (Oxy-4 mini, PreSens, Germany) via a 4 mm tube. Haemolymph O_2 concentration (mg l^{-1}) was measured every 5 s for 10 min. The oxygen probe readings were allowed to stabilise for 5 min before the mean oxygen concentration was calculated for the final 5 min of each 10 min sampling period. The haemolymph pH was determined in a 100 µl sample of pure haemolymph. Haemolymph was extracted from each lobster immediately following $\dot{M}O_2$ measurements and transferred straightaway to plastic needle covers from a 21 G needle stored on ice. The needle covers were connected to the pH needle and a micro fiber optic pH sensor (pH-1 micro, PreSens, Germany) was ejected fully into the sample. pH was measured every 5 s for 10 min and the mean pH was calculated over the final 5 min of each 10 min sampling period to allow the probe readings to stabilise.

6.3.6 Haemolymph biochemistry

Haemolymph samples were extracted from the pericardial cavity of each lobster (see section 6.3.1) following $\dot{M}O_2$ measurements and transferred to eppendorf tubes stored on ice and straightaway diluted with 10% sodium citrate (Sigma, S1804) at a ratio of 1:2 sodium citrate to haemolymph to prevent coagulation. Haemolymph samples were then promptly snap frozen in liquid nitrogen and stored at -80°C until further analysis.

The glucose concentration (mmol l^{-1}) of a 10 µl haemolymph sample was determined in duplicate using an Analox GM7 Micro-Stat Multiassay Analyser (Analox Instruments, London, UK) according to the manufacturers' instructions and protocols (Fearman and Moltschaniwskyj, 2010).

The lactate concentration (mmol l^{-1}) of a 7 µl haemolymph sample was determined in duplicate using an Analox GM7 Micro-Stat Multiassay Analyser (Analox Instruments, London, UK) according to the manufacturers' instructions and protocols (Wilkinson et al., 2008).

Ammonia concentration (mmol l^{-1}) of a 25 µl haemolymph sample was determined using an Analox GM7 Micro-Stat Multiassay Analyser (Analox Instruments, London, UK) according to the manufacturers' instructions and protocols.

The total haemolymph protein concentration (mg ml^{-1}) of a 10 µl sample was determined following a modified method of Bradford (1976) using bovine serum albumin (BSA) as the standard (Sigma-Aldrich, NSW, Australia). Assays were performed in duplicate with the absorbance read using a TECAN Spectro Rainbow Thermo microplate reader (M-Code 2459973, Salzburg, Austria). In a 96-well plate, 5 µl of each unknown protein sample was added before the addition of 250 µl of BCA working reagent. The well plate was placed in the reader, gently mixed, and

absorbance read at 595 nm. Total protein was determined by comparing the absorbance of each unknown sample to the standard curve prepared using the BSA protein standards.

6.3.7 Haemolymph clotting time

The haemolymph clotting time was measured following the method of Jussila et al. (2001) immediately after extraction of 25 µl of pure haemolymph from lobsters. The flow of haemolymph inside a pre-cooled (with ice) plain soda lime glass capillary tube, with an internal diameter of 1.15 mm and length of 75 mm, was measured. Immediately after the haemolymph was inserted, the tube was held vertically with the sample in the upper end. The tube was maintained vertically until gravity forced the haemolymph to the bottom of the tube, after which time the tube was rotated 180°. This was repeated until the haemolymph clotted. The clotting time was defined as the point when the flow of haemolymph ceased and total clotting presumably occurs (Jussila et al., 2001). Samples that were still liquid at 180 s were deemed to have a clotting time of 180 s.

6.3.8 Data analysis

Oxygen consumption rates of lobsters and controls were determined using linear regression of the rate of decline in dissolved oxygen concentration for the final 8 min of every 10 min closed respirometer cycle. Only measurements with regression coefficients (R^2) above 0.95 were used to calculate $\dot{M}O_2$ of juveniles and background $\dot{M}O_2$. Thermal tolerance thresholds of T_p and T_c were determined using the combined shift in trends of R_s and haemolymph O_2 concentration data. Residual plots were used to explore normality and homogeneity of data. Haemolymph samples that had values outside the accurate detection limits of individual analyses were excluded from the

dataset. Exponential regressions were fitted to R_s data and used to describe the relationship between R_s and temperature. Loess trend lines were used to describe the effect of temperature treatments on ammonia-N excretion rate, O:N ratio, and haemolymph parameters. The level of significance for all analyses was determined at $P<0.05$. Data are presented as mean ± standard error (SE) unless stated otherwise. Statistical analyses were performed using SigmaPlot version 12.0 (2011 Systat Software Inc.).

6.4 Results

6.4.1 Oxygen-limited thermal tolerance

The maximum $\dot{M}O_2$ reached at high temperatures indicated the upper T_c threshold at 30°C for both lobster size classes (Fig. 6.1A and 6.2A). However, the low T_c thresholds were not determined in the present study because temperatures were not extended low enough to encounter the onset of anaerobic metabolism at low temperatures. The thermal tolerance window (low T_c to high T_c) was therefore defined between 4°C and 30°C. All lobsters in both size classes died within 2.6 h of the final temperature change in the 32°C treatment and within 1 h of changing the temperature to 34°C in the 34°C and 36°C treatments.

There were sudden changes in the haemolymph O_2 concentration of large lobsters at 12°C and 26°C, indicating the existence of low and high T_p thresholds, respectively, which were used to define the thermal optimum window for large lobsters (Fig. 6.1B). Likewise, dramatic changes in haemolymph O_2 concentration of small lobsters indicated the existence of low and high T_p thresholds at 10°C and 24°C, respectively, which were used to define the thermal optimum window for small lobsters (Fig. 6.2B).

There was a significant exponential relationship between R_s and temperature for large lobsters between 12°C and 30°C (F= 656.73, df 1, 9, P<0.001). The R_s declined dramatically beyond 30°C (high T_c) and increased abruptly below 12°C (low T_p) up until 4°C. Haemolymph O_2 concentration of large lobsters remained relatively constant within the thermal optimum window (12°C to 26°C) but increased below the low T_p threshold. Haemolymph O_2 concentration also increased above the high T_p threshold until the upper T_c threshold was reached at 30°C, where it declined sharply.

There was a significant exponential relationship between R_s and temperature for small lobsters within the thermal optimum window (4°C to 30°C) (F= 115.39, df 1, 13, P<0.001). The R_s then declined sharply above 30°C (high T_c). Haemolymph O_2 concentration of small lobsters fluctuated within the thermal optimum window. Below the low T_p threshold, haemolymph O_2 concentration increased sharply between 10°C and 8°C but decreased above the high T_p threshold and continued decreasing to the T_c threshold at 30°C.

6.4.2 Temperature coefficients

The Q_{10} values of R_s in the thermal optimum window were 2.7 for large and small lobsters (Table 6.1). The Q_{10} values were 2.7 for large lobsters and 2.4 for small lobsters in the thermal tolerance window. However, below the low T_p threshold Q_{10} values of R_s were higher for both large (15.9) and small (8.6) lobsters. The Q_{10} values were also much lower above the high T_p threshold for large (0.5) and small (1.3) lobsters.

6.4.3 Ammonia-N excretion rate

Ammonia-N excretion rates of large lobsters progressively increased within the thermal optimum window either side of the control temperature of 20°C (Fig. 6.3A). Ammonia-N excretion rates decreased thereafter, below the low T_p and above the high T_c thresholds. The atomic O:N ratio slightly increased with temperature within the thermal optimum window for large lobsters and steadily decreased above the high T_p range (Fig. 6.3B).

Ammonia-N excretion rates of small lobsters peaked around the control temperature (20°C) and decreased either side of this temperature within the thermal optimum window (Fig. 6.4A). Ammonia-N excretion rates then increased between

the upper T_p and T_c before decreasing above the T_c threshold. Ammonia-N excretion rates remained constantly low below the low T_p threshold. The atomic O:N ratio for small lobsters gradually increased above 20°C and increased sharply below 20°C within the thermal optimum window (Fig. 6.4B). The O:N ratio then continually declined outside this temperature range (i.e. outside the T_p thresholds).

6.4.4 Haemolymph measurements

Within the thermal optimum window, haemolymph pH increased sharply above and below 16°C for large lobsters (Fig. 6.3C). Haemolymph pH then declined outside the T_p thresholds. This trend was also observed for haemolymph pH in small lobsters (Fig. 6.4C).

Haemolymph clotting times of large lobsters increased above and below 18°C within the thermal optimum window and continued to increase beyond the T_p thresholds except for a decrease between 8°C and 4°C (Fig. 6.3D). Haemolymph clotting times of small lobsters fluctuated between consistently high values within the thermal optimum window and above the upper T_c threshold, but decreased sharply below the low T_p threshold (Fig. 6.4D).

Haemolymph glucose concentration of large lobsters remained constant within the thermal tolerance window (4°C to 30°C), averaging 2.31 ± 0.19 mmol l^{-1}, but increased dramatically above the high T_c threshold to average 8.42 ± 1.27 mmol l^{-1} between 32°C and 36°C (Fig. 6.3E). Haemolymph glucose concentration of small lobsters remained constant between 4°C and 24°C (mean 2.64 ± 0.38 mmol l^{-1}), but steadily increased above the high T_c threshold to 3.92 ± 0.19 mmol l^{-1} between 32°C and 36°C (Fig. 6.4E).

Haemolymph lactate concentrations remained stable in large lobsters within the thermal tolerance window, averaging 1.06 ± 0.12 mmol l^{-1}, but increased sharply

above the T_c threshold at 30°C to an average of 23.24 ± 0.78 mmol l^{-1} between 32°C and 36°C (Fig. 6.3F). Haemolymph lactate concentrations of small lobsters followed a similar trend, averaging 1.25 ± 0.15 mmol l^{-1} within the thermal tolerance window, before increasing sharply above the high T_c threshold at 30°C to average 21.09 ± 0.97 mmol l^{-1} between 32°C and 36°C (Fig. 6.4F).

Haemolymph ammonia concentration peaked at 18°C for large lobsters and declined gradually either side of this temperature within the thermal optimum window and continued to decline outside this range to 15.0 and 0.0 mmol l^{-1} beyond the high T_p and low T_p thresholds, respectively (Fig. 6.3G). Haemolymph ammonia concentration of small lobsters mostly displayed a decreasing trend from 6°C to 36°C, ranging between 0.0 and 639.0 mmol l^{-1} (Fig. 6.4G).

Haemolymph protein concentration of large lobsters within the thermal optimum window showed a decreasing trend above and below the control temperature of 20°C, ranging from 3.86 to 8.53 mg ml^{-1} (Fig. 6.3H). Haemolymph protein concentration continued to decline beyond the low and high T_p thresholds, but increased above the upper T_c threshold. Haemolymph protein concentration of small lobsters remained reasonably constant within the thermal optimum window, fluctuating between 1.84 and 4.20 mg ml^{-1} (Fig. 6.4H). Haemolymph protein concentration declined steadily below the low T_p threshold and gradually increased above the high T_c threshold.

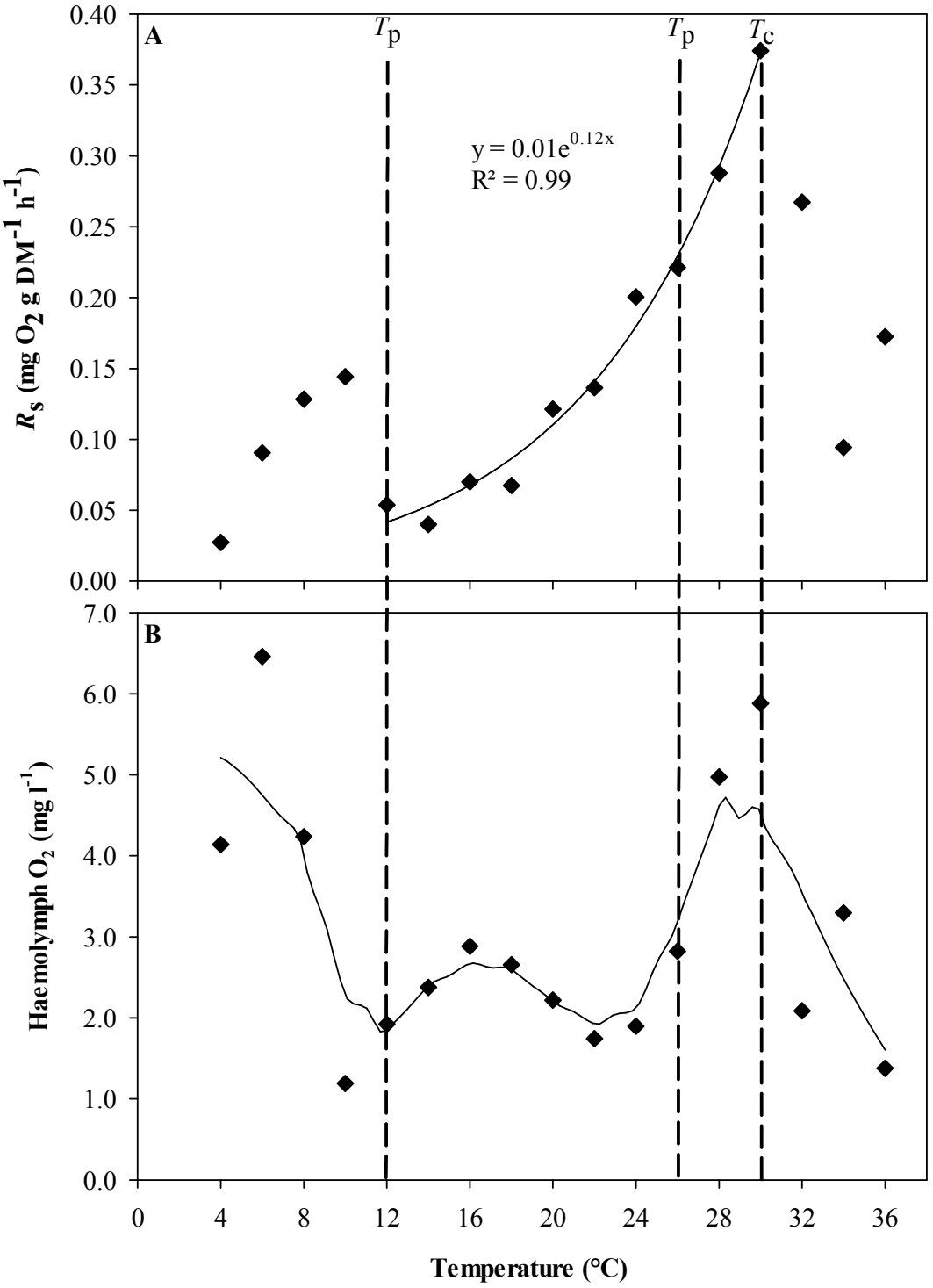

Figure 6.1. Oxygen-limited thermal tolerance of large juvenile *Sagmariasus verreauxi* co-determined by investigation of: (A) standard metabolic rate (R_s) showing exponential regression (solid line) with equation and; (B) haemolymph O_2 concentration showing loess trend (solid line) at different temperatures. Dashed lines indicate threshold temperatures of low and high *pejus* temperatures (T_p) at 12°C and 26°C, respectively, and the upper critical temperature (T_c) at 30°C.

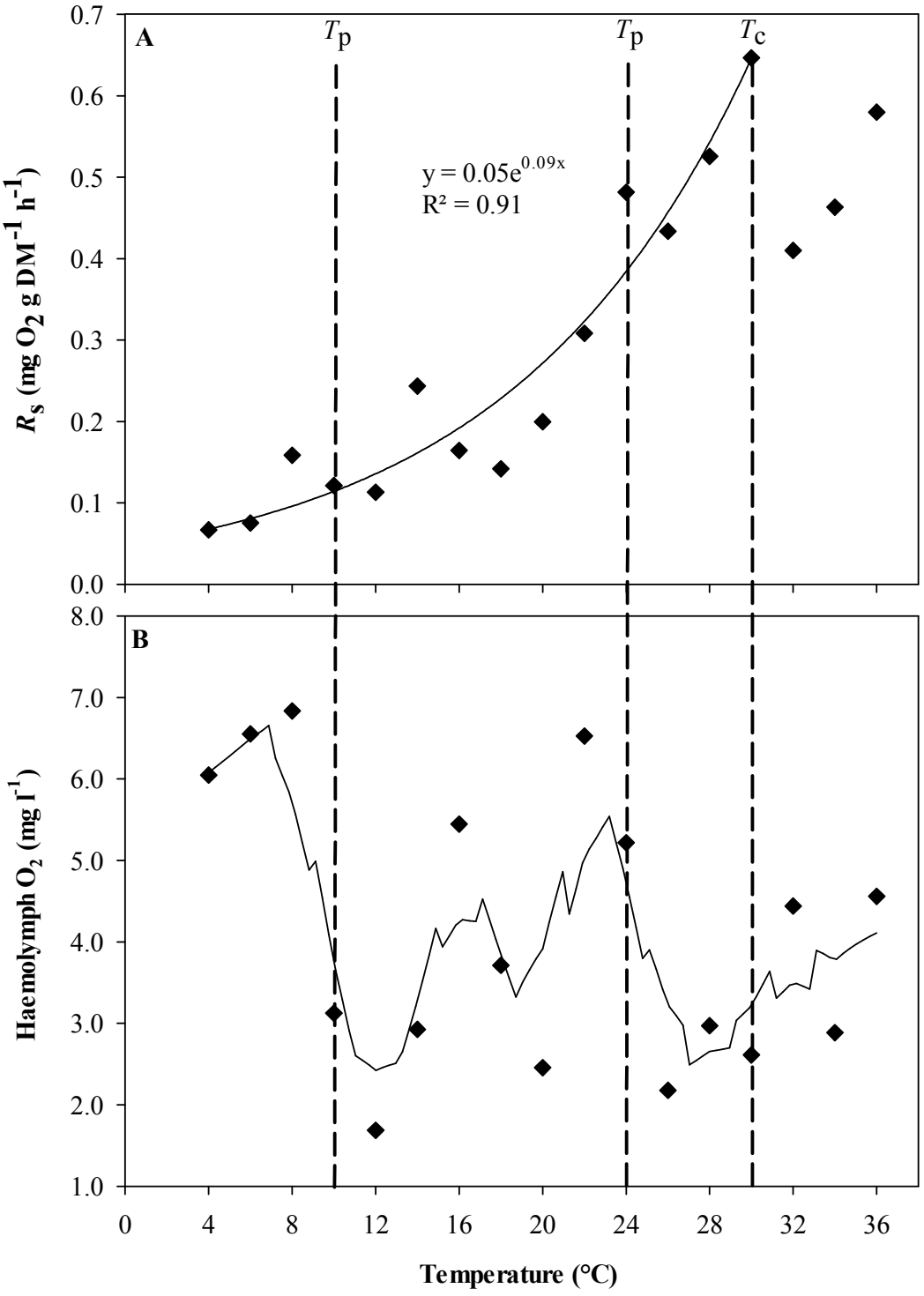

Figure 6.2. Oxygen-limited thermal tolerance of small juvenile *Sagmariasus verreauxi* co-determined by investigation of: (A) standard metabolic rate (R_s) showing exponential regression (solid line) with equation and; (B) haemolymph O_2 concentration showing loess trend (solid line) at different temperatures. Dashed lines indicate threshold temperatures of low and high *pejus* temperatures (T_p) at 10°C and 24°C, respectively, and the upper critical temperature (T_c) at 30°C.

Table 6.1. Temperature coefficients (Q_{10}) for standard metabolic rate (R_s) (mg O_2 g DM^{-1} h^{-1}) of large and small juvenile *Sagmariasus verreauxi* between different temperature thresholds.

	Below low T_p	**Thermal optimum window**	**Thermal tolerance window**	**Above high T_p**
Temperature range	4°C-10°C	12°C-26°C	4°C-30°C	28°C-36°C
Large lobsters	15.9	2.7	2.7	0.5
Temperature range	4°C-8°C	10°C-24°C	4°C-30°C	26°C-36°C
Small lobsters	8.6	2.7	2.4	1.3

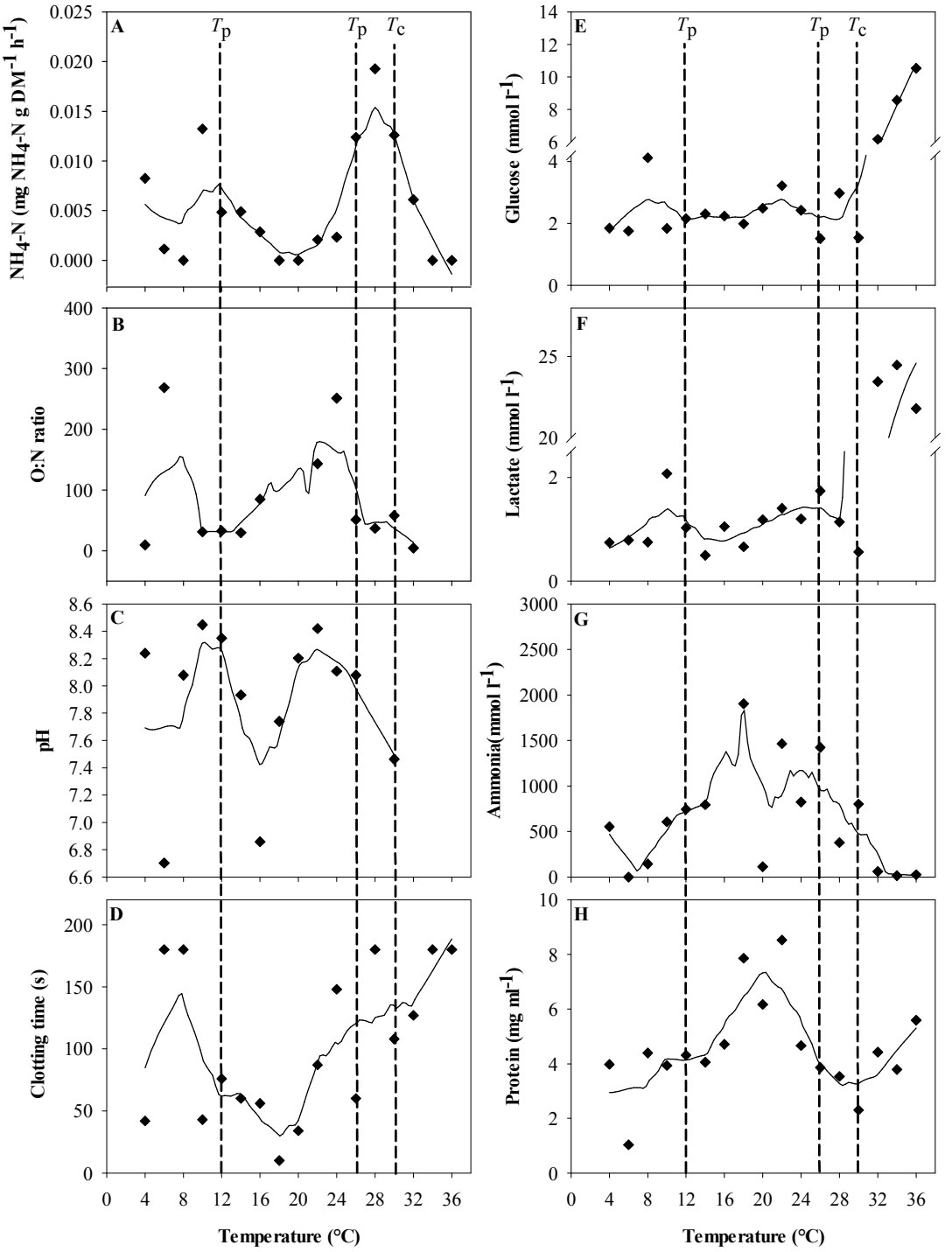

Figure 6.3. The effect of temperature on: (A) ammonia-N excretion rate; (B) atomic O:N ratio; (C) haemolymph pH; (D) haemolymph clotting time; (E) haemolymph glucose concentration; (F) haemolymph lactate concentration; (G) haemolymph ammonia concentration and; (H) haemolymph protein concentration trends of large juvenile *Sagmariasus verreauxi*. Solid lines represent fitted loess trends. Dashed lines indicate threshold temperatures of low and high *pejus* temperatures (T_p) at 12°C and 26°C, respectively, and the upper critical temperature (T_c) at 30°C.

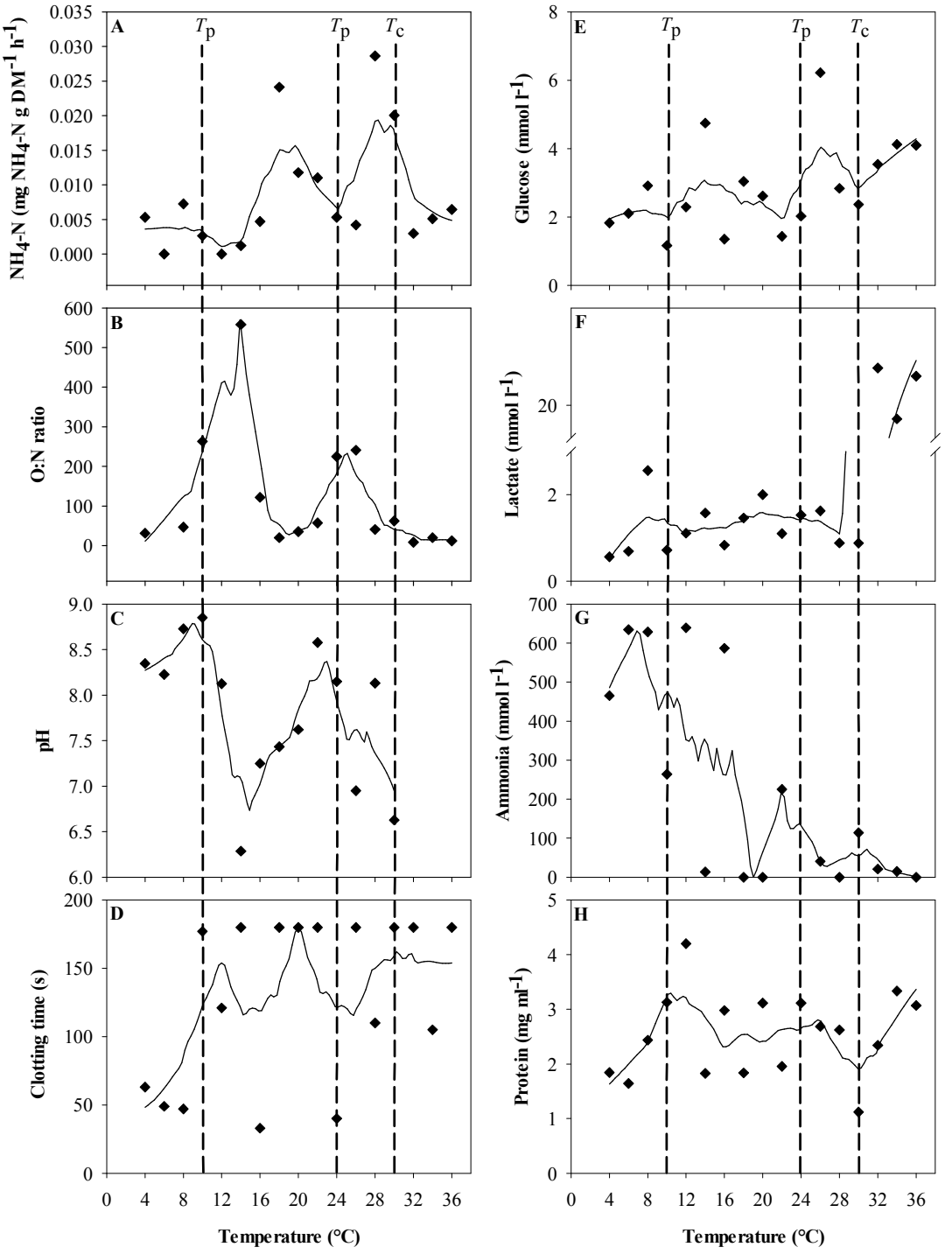

Figure 6.4. The effect of temperature on: (A) ammonia-N excretion rate; (B) atomic O:N ratio; (C) haemolymph pH; (D) haemolymph clotting time; (E) haemolymph glucose concentration; (F) haemolymph lactate concentration; (G) haemolymph ammonia concentration and; (H) haemolymph protein concentration trends of small juvenile *Sagmariasus verreauxi*. Solid lines represent fitted loess trends. Dashed lines indicate threshold temperatures of low and high *pejus* temperatures (T_p) at 10°C and 24°C, respectively, and the upper critical temperature (T_c) at 30°C.

6.5 Discussion

6.5.1 Thermal tolerance

Differences in thermal tolerance windows between two size classes of juvenile *S. verreauxi* were determined using measurements of R_s and haemolymph O_2 concentration. Thermal tolerance patterns observed in both juvenile size classes support the concept that thermal tolerance is oxygen and capacity limited (Frederich and Pörtner, 2000; Pörtner et al., 2005; Storch et al., 2011). The thermal optimum window was similar for both size classes of juvenile *S. verreauxi* (14°C) and is much wider than in several other crustacean species (Frederich and Pörtner, 2000; Storch et al., 2011), which suggests they have a greater capacity to adapt to climate change. Smaller lobsters preferred a slightly cooler optimum window (10°C-24°C) than larger animals (12°C-26°C), which agrees with recent observations that small juvenile *S. verreauxi* are already expanding their distribution southward to cooler waters of Tasmania (Johnson et al., 2011).

Within the thermal tolerance window set by upper and lower T_c, R_s of inactive, unfed animals normally increases exponentially with increasing temperature (Pörtner et al., 2005; Melzner et al., 2006, Wittmann et al., 2008). Assuming that the exponential relationship between R_s and temperature indicates thermal tolerance limits, the upper T_c in both size classes of juvenile *S. verreauxi* is 30°C. There was also a clear reduction in R_s beyond the upper T_c, and eventuated in the death of all lobsters at temperatures above 30°C. These results confirm that respiratory failure, sometimes in combination with circulatory failure, is most likely the cause of temperature-induced death in invertebrates (Pörtner et al., 2005) and prolonged

exposure to temperatures beyond T_c thresholds will inevitably result in death unless thermal acclimation occurs (Zielinski and Pörtner, 1996; Sommer et al., 1997). In the present study, temperature was changed rapidly over a wide range, which minimised thermal acclimation trends that cause a shift in thermal thresholds. The estimates of T_c thresholds in the present study are therefore considered to be accurate for this species. The substantial increase in R_s between 10°C and 12°C of the larger lobsters may also indicate that a thermal limitation or T_p has been reached (Frederich and Pörtner, 2000). However, the thermal optimum window of marine organisms correlates to their aerobic scope and a reduction in aerobic scope is commonly used to define the onset of T_p temperature thresholds (Pörtner et al., 2005). Since aerobic scope was not assessed in the present study, measurements of R_s recorded here cannot be used exclusively to determine T_p thresholds.

Accordingly, haemolymph O_2 concentration was used in conjunction with R_s to identify thermal tolerance thresholds. The shift from a thermal range with maximum haemolymph O_2 to progressive hypoxic haemolymph O_2 during an increase or decrease in temperature is indicative of the lower and upper T_p thresholds, which are within the range covered by T_c thresholds (Pörtner, 2001). A drop in haemolymph O_2 also indicates excessive oxygen demand (or reduction in oxygen availability) at high temperatures or decreased aerobic capacity of mitochondria at low temperatures (Pörtner, 2001). However, while declining temperature reduces the capacity for oxygen supply, it also provides increased oxygen solubility and, consequently, elevated concentrations of ambient and arterial oxygen (Pörtner, 2010). This increased oxygen solubility may also be paralleled by a reduction in metabolic rate (as seen in smaller lobsters), which could explain why haemolymph O_2 did not decrease below the low T_p thresholds in both sizes of lobsters in the present study since $\dot{M}O_2$

suggested oxygen availability was not limited and, in fact, increased in larger animals. This increase in haemolymph O_2 of *S. verreauxi* is consistent with recent findings that a reduction in haemolymph O_2 from exposure to colder temperatures is less prolific in other temperate crustaceans such as *Hyas araneus* and *Cancer pagurus* (Metzger et al., 2007; Walther et al., 2009). This increase in haemolymph O_2 may also be attributed to an increase in haemocyanin oxygen-affinity, which greatly improves lobsters ability to extract and utilise oxygen and has been observed previously during periods of hypoxic stress in *Homarus gammarus* (Taylor and Whiteley, 1989). Because both size classes of *S. verreauxi* reached their highest haemolymph O_2 concentrations at low temperatures (6°C and 8°C for large and small lobsters, respectively), this may indicate that this species does not experience oxygen limitation at cold temperatures, at least in the central organs in the pericardial cavity as reported in *H. araneus* (Walther et al., 2009). Pörtner (2001) reported a reduction in haemolymph O_2 occurs when ventilation rates decrease above high temperature thresholds, suggesting an increase in oxygen demand is no longer compensated for. This is consistent with the present study where reduced $\dot{M}O_2$ accounted for a decrease in haemolymph O_2 above 30°C in large *S. verreauxi*.

Most crustaceans have a Q_{10} between 2 and 3 within the thermal tolerance window (Katsanevakis et al., 2007), while values approaching 8 are usually characteristic of animals exposed to temperatures outside their thermal tolerance range (Johnston et al., 1991). This is consistent with the present study where Q_{10} values for R_s were 2.7 for both lobster size classes within the thermal optimum window and 2.7 (large lobsters) and 2.4 (small lobsters) within the thermal tolerance window (4°C-30°C). However, Q_{10} values were very high, being 15.9 (large lobsters) and 8.6 (small lobsters) below the low T_p threshold.

6.5.2 Energy utilisation and ammonia-N excretion rate

Atomic O:N ratios derived from ammonia-N excretion rates are used to assess changes in energy substrate utilisation under various conditions in ammoniotelic animals (Corner and Cowey, 1968; Carter and Brafield, 1991). An O:N ratio of 9.2 indicates only protein is being metabolised (if under aerobic conditions) where as a ratio above 18.5 indicates more than half the oxygen is being used to metabolise non-protein substrates (Brafield, 1985). In the present study, protein catabolism increased above the high T_p threshold as shown by an O:N ratio shift from a mean of 90 to 33 and 182 to 64 in large and small lobsters, respectively. This trend was also observed in small lobsters below the low T_p threshold, with an average O:N ratio of 39. This shift in the O:N ratio is consistent with previous observations that protein dominated metabolism (low O:N ratio) in crustaceans is generally an indication of stressful conditions (Pillai and Diwan, 2002). The O:N ratios in the present study are still above 18.5 and suggest less than a quarter of oxygen was used to metabolise proteins. This, however, assumes aerobic metabolism of proteins to ammonia, although some anaerobic metabolism was occurring (see below). Because O:N ratios are specific to aerobic metabolism, O:N values estimated during anaerobic metabolism are not an accurate depiction of energy substrates.

Lobsters remove ammonia primarily by diffusion across the gills via the respiratory current into the surrounding water (Vermeer, 1987). If respiratory functioning becomes limited or fails, however, this route is eliminated and ammonia is accumulated in the haemolymph (Huang and Chen, 2001). The concentration of this waste product in the haemolymph has therefore been reported to rise in response to increased ambient ammonia in the water or emersion, both of which impair gill ammonia excretion (Vermeer, 1987; Chen et al., 1994; Scmitt and Uglow, 1997a;

1997b). Haemolymph ammonia concentration though decreases in aquatic crustaceans in response to stress when immersed in water (Regnault and Lagardere, 1983; Regnault, 1987). This is consistent with the present study, where haemolymph ammonia concentrations decreased above the upper T_c in both size classes of lobsters.

6.5.3 Thermal stress

Thermal stress in crustaceans is generally characterised by an accumulation of metabolic by-products such as ammonia and lactate (Schmitt and Uglow, 1997b). Lactate, in particular, is the main end-product of anaerobic metabolism in crustaceans (Spicer et al., 1990). Elevated concentrations of lactate are consequently indicative of anaerobic metabolism and most likely results from an inability to maintain an adequate supply of oxygen to tissues (Spicer et al., 1990; Ridgway et al., 2006). In *S. verreauxi*, haemolymph lactate levels were similar within the thermal tolerance window for both large and small lobsters, but temperatures beyond 30°C (T_c) caused a clear rise in haemolymph lactate. This is consistent with Ryan (1995), who reported that haemolymph lactate levels in *Pagothenia borchgrevinki* increased significantly above the upper T_c threshold. It should be noted that lactate becomes compartmentalised in specific tissues and whole-body analysis provides a more effective measure of lactate (Ocampo et al., 2003) and the time scale of lactate release from muscles is unclear (Full and Herreid, 1984). The majority of lactate compartmentalised in lobster muscle tissues, however, is formed from anaerobic metabolism during tail-flipping, which is used as an escape response (Jimenez et al., 2008). Given that lobsters in the present study were not chased during capture and were restrained during haemolymph extraction, haemolymph (as opposed to whole-body) concentrations should therefore accurately represent lactate levels of juvenile *S. verreauxi*.

Haemolymph clotting time has been reported as a useful indicator of stress in lobsters (Jussila et al., 2001). Haemolymph clotting in crustaceans is an important defensive mechanism of the immune system to protect against potential pathogens (Muta and Iwanaga, 1996) and, therefore, maybe a useful indicator of the overall health of an animal. The haemolymph clotting times determined in large juvenile *S. verreauxi* are consistent with those reported in *Panulirus cygnus*, with higher clotting times observed at increasingly stressful temperatures (Jussila et al., 2001). However, this trend was not observed in smaller lobsters; over half the haemolymph samples did not clot within 180 s. Haemolymph clotting times have only been measured in very few previous studies (Battelle and Kravitz, 1978; Gondko et al., 1981; Smith et al., 1995; Jussila et al., 2001) and although the method used in the present study was designed to clot haemolymph within 180 s, most observations on clotting times for smaller crustaceans have been a few minutes (Battelle and Kravitz, 1978; Gondko et al., 1981; Smith et al., 1995). Clotting times for smaller lobsters should therefore be monitored for longer because their haemolymph could take longer to clot compared to larger lobsters, possibly due to differences in haemolymph constituents such as presence of bacteria and differences in the proportion of hyalinocytes and semigranulocytes (Jussila et al., 2001).

Haemolymph glucose levels are known to become elevated after various forms of stress, including exposure to chronic and acute changes in temperature (Kindle and Whitmore, 1986; van Dijk et al., 1993; Staurnes et al., 1994). Haemolymph glucose concentrations are related to the mobilisation of energy stores during stressful conditions as a source of energy to fuel anaerobic metabolism (Lorenzon et al., 2007). Haemolymph glucose levels in *S. verreauxi* remained reasonably stable within the thermal tolerance window, however, a marked increase in haemolymph glucose above

the T_c threshold was observed in both lobster size classes. The most likely explanation for this is an increase in adrenaline and cortisol (common stress hormones), which may cause hyperglycaemia in crustaceans (Radford et al., 2005; Saydmohammed and Pal, 2009). Hyperglycaemia also develops more rapidly with increasing temperature (Schmitt and Uglow, 1997a). In crustaceans, haemolymph glucose concentrations are regulated at a steady-state by crustacean hyperglycaemic hormone (CHH), with a rapid turnover between uptake and release by tissues (Speck and Ulrich, 1972; Herz-Hübner et al., 1973; Urich and Speck, 1973). Elevation in glucose concentrations are therefore usually followed by a rapid return to baseline levels (Santos et al., 1988; Santos and Keller, 1993a; Zou and Bonvillain, 2003). The increase above steady-state in the present study would suggest a failure of the homeostatic processes controlling haemolymph glucose occurred above T_c thresholds in *S. verreauxi*. Since glucose is used as an energy source during anaerobic metabolism, increased glucose concentrations also result in the production of lactate (Lorenzon et al., 2007).

As a consequence of increased haemolymph lactate concentration, the haemolymph pH was also altered, but may not be the main component controlling pH. It has been shown that when crustaceans are re-immersed after a period of aerial exposure, the pH increases dramatically even though lactate concentration remain high, indicating that the accumulation of CO_2 in the haemolymph is pivotal in determining the pH (Truchot, 1975; Taylor and Wheatley, 1981; Gilmour and Perry, 1994). Marine invertebrates regulate the pH of their body fluids, including haemolymph, so that the level of protonation of alpha imidazole groups is maintained despite changes in body temperature (Reeves, 1972). This process is called alpha-stat regulation and is crucial to maintaining the structural integrity of proteins during

temperature changes within the thermal tolerance window (Hochachka and Somero, 1984; Sommer et al., 1997). Results of haemolymph pH observed here for both sizes of *S. verreauxi* suggested that pH regulation began to shift at the low and high T_p thresholds. This is comparable to results found in *Pachycara brachycephalum* where T_p correlated to a shift in alpha-stat pH regulation (van Dijk et al., 1999; Mark et al., 2002). These results confirm previous suggestions that the functional capacities of many physiological processes are set at an optimum within the thermal optimum window and may become limited outside this temperature range. A possible explanation for this shift in pH regulation might be due to the temperature sensitivity of ion channels or a change in the relationship between membrane permeability and compensatory ion exchange (Mark et al., 2002).

In the present study, an increase in haemolymph protein concentration was observed above the upper T_c threshold. This increase in haemolymph protein may be related to a higher energy demand and the regulatory responses at stressful temperatures (Lorenzon et al., 2007). As a consequence of rising temperature, the overall metabolism of crustaceans is increased, requiring addition energy intake (Pascual et al., 2003), which may result in proteins being used to repair membranes (Lorenzon et al., 2007). Haemolymph proteins, along with the carbonate-bicarbonate system, have an extensive physiological role in acid-base regulation (Wheatly and Henry, 1992). The majority of protein in the haemolymph however is associated with oxygen transport (haemocyanin) or host-defence reactions (haemocytes) (Lorenzon et al., 2007). The oxygen-binding molecule, haemocyanin, usually accounts for the largest proportion of haemolymph protein (Depledge and Bjerregaard, 1989). Lorenzon et al. (2007) observed that increases in haemolymph protein concentrations in *Macrobrachium rosenbergii* were the result of an increase in haemocyanin during

the process of physiological compensation. Even though haemocyanin is disabled in response to some stressors, such as hypoxia, compensatory synthesis of additional haemocyanin in response to oxygen deprivation has also been reported (Hagerman and Pihl Baden, 1988; Hagerman et al., 1990). Compensatory synthesis of haemocyanin may explain why the haemolymph protein concentration in juvenile *S. verreauxi* increased slightly above the upper T_c threshold, because oxygen availability was limited beyond this threshold.

6.5.4 Conclusion

The present study is the first to demonstrate evidence of oxygen related thermal tolerance in juvenile spiny lobsters. This thermal tolerance was characterised by the transition to anaerobic metabolism at temperatures above the T_c threshold (above 30°C). Both size classes of juvenile *S. verreauxi* have a wider thermal optimum window (14°C) than several other crustacean species, which suggests they have a greater capacity to adapt to climate change. Smaller lobsters preferred a slightly cooler optimum window (10°C-24°C) than larger lobsters (12°C-26°C), indicating they have an increased ability to continue their distribution further south if they are confronted with temperatures approaching T_c and even T_p thresholds in the wild. This expanded distribution may have a large impact on benthic community structures and dynamics, which is an important consideration for future research.

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GENERAL DISCUSSION

Chapter VII

7.1 Overview

This thesis examined in detail the physiological responses to different environmental and culture conditions of *Sagmariasus verreauxi* through ontogeny. A number of exogenous and endogenous factors will affect the physiological processes of spiny lobster species and the current research advances our understanding of metabolism in one species and how metabolism in this species relates to other animals. Of all these parameters, body mass is the major endogenous factor influencing physiological processes (Bishop and Torres, 1999; Anger, 2001). Therefore, the purpose of this chapter is to overview stage-specific effects of body mass on the energetic demands of *S. verreauxi* during complete ontogeny through collating the routine metabolic rate (R_r), ammonia-N excretion rate, and O:N ratio data for phyllosoma generated in Chapters 3 with additional data obtained from different sized juvenile lobsters. The allometric relationship between R_r and body mass for *S. verreauxi* was compared to 2824 measurements of R_r and body mass from 938 vertebrate species recorded by White et al. (2006) to determine if this relationship scales with the commonly cited 0.75 (3/4) and 0.67 (1/3) mass dependent scaling exponents. The effect of juvenile body mass on metabolic scope was also modelled in order to predict differences in the ability of different sized *S. verreauxi* juveniles to perform physiological functions.

7.2 Routine metabolic rate

This research demonstrated that body mass and activity have a significant effect on the oxygen consumption rate ($\dot{M}O_2$) and energy utilisation of *S. verreauxi* during ontogeny. Mass-specific R_r of *S. verreauxi* was significantly higher following hatch-out compared to all other life stages (Fig. 7.1A). The higher mass-specific R_r of

early stage phyllosoma compared to subsequent stages may be caused by increased swimming activity due to reduced buoyancy of phyllosoma associated with their body composition (Jeffs et al., 1999); the reduced size of appendages; and the photopositive response of instar 1 phyllosoma (Ritz, 1972). Higher R_r in early stages may also be attributed to a decrease in the surface area: volume ratio of lobsters limiting gas exchange (Childress and Somero, 1990); and the transitions in lifestyle from planktonic development (Lemos and Phan, 2001) through to the benthic juvenile stage. These changes in morphology and lifestyle lead to an overall reduction in oxygen demand throughout development (Capuzzo and Lancaster, 1979; Chu and Ovsianico-Koulikowsky, 1994). The current research also demonstrated an increase in mass-specific R_r during later phyllosoma stages which does not commonly occur in other crustaceans and may be unique to *S. verreauxi* phyllosoma. This increase in R_r occurred at instars 15 and 17 and corresponds with the development of gills and pleopods, which may allow greater gas exchange (Spicer, 1995). Because the pleopods in late stage phyllosoma are nearly as well developed as in pueruli, these may also be used for swimming (Chiswell and Booth, 1999) and result in an increase in activity. Elevated R_r in late stage phyllosoma may also be explained by the high energy requirements and oxygen demand of phyllosoma in preparation for metamorphosis (Lemmens, 1994; Jeffs et al., 2001). Conversely, the lower mass-specific R_r in juvenile *S. verreauxi* may be characteristic of the reduced activity levels typical of the benthic stages of spiny lobsters (Crear and Forteath, 2000).

7.3 Ammonia-N excretion rate

Mass-specific ammonia-N excretion rates have not been measured during the complete ontogeny of any crustacean species. Mass-specific ammonia-N excretion rates generally decreased throughout larval development of *S. verreauxi*, apart from a

large increase at instar 17, and continued to decrease in juvenile lobsters (Fig. 7.1B). Significant ontogenetic declines in ammonia-N excretion rates have been reported previously during early phyllosoma stages (Bermudes and Ritar, 2004; Bermudes et al., 2008) and juvenile stages of *Jasus edwardsii* (Crear and Forteath, 2002) and *Panulirus homarus rubellus* (Kemp et al., 2009) and in *Macrobrachium rosenbergii* (Agard, 1999). Ammonia-N excretion rates are representative of protein catabolism and previous research has indicated that low ammonia-N excretion rates are due to preferential partitioning of dietary protein to somatic growth rather than catabolism (Ikeda et al., 2000; 2011). The rate of ammonia-N excretion as a percentage of total nitrogenous excretion can also change with body mass (Quarmby, 1985), which may affect the relationship between ammonia-N excretion rate and body mass (Crear and Forteath, 2002).

The observed increase in ammonia-N excretion rates of final instar phyllosoma may be unique to *S. verreauxi*, which could reflect species-specific differences in energy substrates between crustaceans (Ikeda et al., 2011). The increase in ammonia-N excretion rate of instar 17 phyllosoma was also characterised by a decrease in the O:N ratio (see Chapter 3), which indicates that there is a shift towards higher protein catabolism prior to metamorphosis possibly due to the preferential storage of lipid as an energy reserve during the non-feeding puerulus stage (Jeffs et al., 1999; Jeffs et al., 2001). In contrast to the final stage, the O:N ratio tended to increase throughout development, which may be associated with shifts in feeding behaviour and food assimilation (Omori, 1979; Ikeda, 1984; 1985; Matsuda et al., 2009). For example, later stage phyllosoma consume prey higher in lipid (Nichols et al., 2001; Jeffs et al., 2004; Johnston et al., 2004). In summary, the O:N ratio indicated that larval development of *S. verreauxi* is predominantly based on protein catabolism, further supporting observations that phyllosoma accumulate lipid reserves

to fuel metamorphosis and the puerulus stage (Jeffs et al., 1999; Jeffs et al., 2001), whereas juvenile development is characterised by lipid catabolism (Fig. 7.1C). Lipid catabolism by juveniles is likely due to the high importance of protein for tissue growth and maintenance in juvenile spiny lobsters (Johnston et al., 2003; Ward et al., 2003).

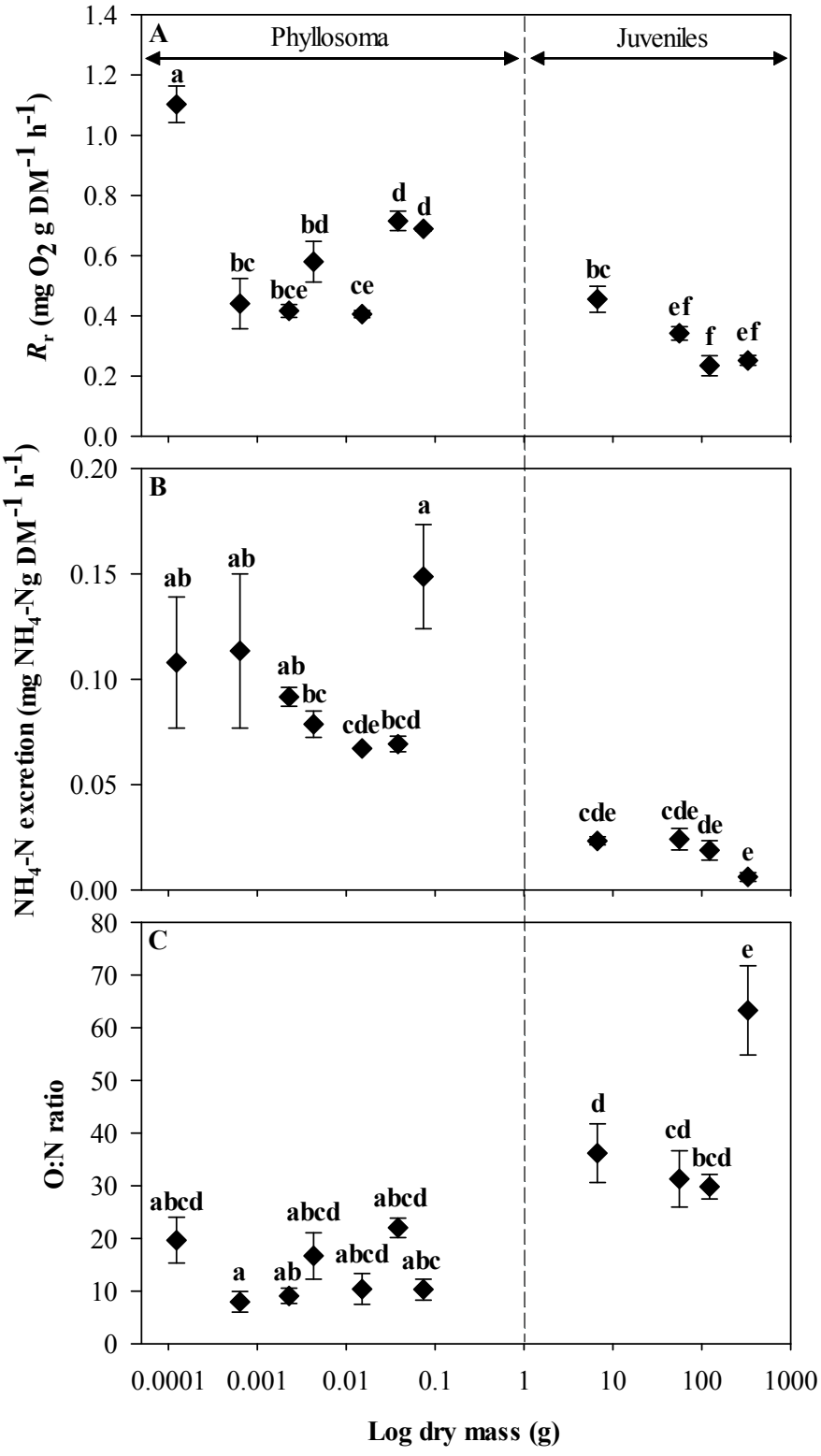

Figure 7.1. Ontogenetic changes in: (A) routine metabolic rate (R_r); (B) ammonia-N excretion rate and; (C) atomic O:N ratio of *Sagmariasus verreauxi* phyllosoma (instar 1 to 17) and juveniles plotted against log of dry mass (DM). Data to the left of the dashed line correspond to phyllosoma and data on the right correspond to juveniles. Data points bearing different superscripts are significantly different (P<0.05). Values are mean (±SE).

7.4 Body mass

Body size has a substantial influence on physiological processes, particularly through larval development (Bishop and Torres, 1999; Anger, 2001). The allometric relationship between metabolic rate and body mass is well-documented in aquatic organisms, but is a poorly understood concept in animal physiology (White et al., 2006). For example, the use of a universal scaling exponent for different animals is still being questioned (Dodds et al., 2001; Bokma, 2004; Kozlowski and Konarzewski, 2004; White et al., 2006). In general, scaling exponents for this allometric relationship have been summarised by several authors and range between 0.5 and 1.0 for animals of different taxonomic groups (Prosser, 1973; Withers, 1992; White et al., 2006). The scaling exponent has recently been shown to change during ontogeny (Moran and Wells, 2007). Development had a profound effect on the scaling of R_r in *S. verreauxi*, as phyllosoma (0.97; see Chapter 3, Fig. 3.4; R_r for Low Density) showed elevated scaling exponents compared with juvenile stages (0.83; Fig. 7.2; R_r). To my knowledge, scaling exponents of decapod crustaceans have not previously been examined during complete ontogeny. However, the elevated scaling exponents of phyllosoma support the general model of R_r during ontogeny in teleosts elucidated by Post and Lee (1996).

Elevated scaling exponents during larval development have previously been attributed to either increased growth rates or high protein turnover (Weiser, 1991; 1995; Glazier, 2005), which increases metabolic rates due to the high energetic cost of biosynthesis and growth. Juveniles and adults grow more slowly and, therefore, have a lower scaling exponent (Glazier, 2005). Even though the growth rates of phyllosoma are not as high as most other crustacean larvae due to their extended larval phase, they are still comparatively much higher than juvenile or adult stages.

The rapid growth of larval animals is accompanied by rapid development of organs with high metabolic activity, which increases metabolic rate (Oikawa et al., 1991). Elevated scaling exponents during larval development may therefore also be due to the temporally variable development of metabolically active tissues and organs (Oikawa et al., 1991).

Increases in respiratory surface area during the larval phase elevate the scaling exponent as larvae shift from diffusive respiration to the use of gills (Kamler, 1992; Post and Lee, 1996). A shift in the scaling exponent could occur if gill surface area increased in relation to gill or larval volume during larval development; total gill surface area decreased in relation to larval volume throughout development or; respiratory surfaces were limiting to R_r throughout development (Post and Lee, 1996). In phyllosoma, this shift may occur to some extent in later larval stages with the appearance of gills, or as a sudden shift with the hardening of the exoskeleton and with development of fully functional gills in juvenile lobsters.

Although my research supports previous observations (Post and Lee, 1996; Moran and Wells, 2007) that the scaling exponent varies during ontogeny, there is a discrepancy as to whether the scaling exponent changes suddenly or gradually (Moran and Wells, 2007). Oikawa et al. (1991) and Post and Lee (1996) suggested a biphasic relationship where the scaling exponent changes from isometry during larval development to allometry during the juvenile phase, which may be associated with an ontogenetic change in the surface area to volume ratio of respiratory organs (Moran and Wells, 2007). In contrast, Bochdansky and Legget (2001) derived a single metabolic model and suggested a curvilinear relationship between mass and metabolic rate where the mass exponent decreases gradually and continuously during ontogeny. Exactly which model is most appropriate may be questioned because there is currently no biological basis to debate which is the more accurate. This is further complicated

by the fact that the scaling exponent may vary with many factors, including mass range, level of activity, and development stage (Weiser, 1995; Lovegrove, 2000; Hochachka and Somero, 2002; Hunt von Herbing, 2005; Rombough, 2006).

Bridges and Brand (1980) suggested that crustacean species with a large mass range (such as lobsters) have higher scaling exponents, which appears to be the case in *S. verreauxi*. This indicates that $\dot{M}O_2$ is more dependent on body mass in larger animals because a larger proportion of their total mass may be used for structural support (Bridges and Brand, 1980), such as the exoskeleton in lobsters. Activity influences metabolic scaling exponents of *S. verreauxi* juveniles, with exponents of 0.91 for standard metabolic rate (R_s) and 0.81 for active metabolic rate (R_{active}) (Fig. 7.2). Higher activity levels tend to reduce the scaling exponent, therefore, differences in scaling exponents between studies may be due to respirometry techniques causing increased activity (Bridges and Brand, 1980) making it important to consider differences in respirometry methods when comparing scaling exponents. Since phyllosoma are more active, which is characteristic of planktonic larvae (Buskey, 1998), compared to relatively inactive benthic juveniles (Crear and Forteath, 2000), the scaling exponent should be lower in phyllosoma. However, this was not the case in the present study where the exponent observed in phyllosoma was higher than for juvenile development. Level of activity may therefore be less important than mass range and developmental stage in determining the scaling exponent. Further research on the effect of R_{active} on the scaling exponent of phyllosoma is required to confirm this.

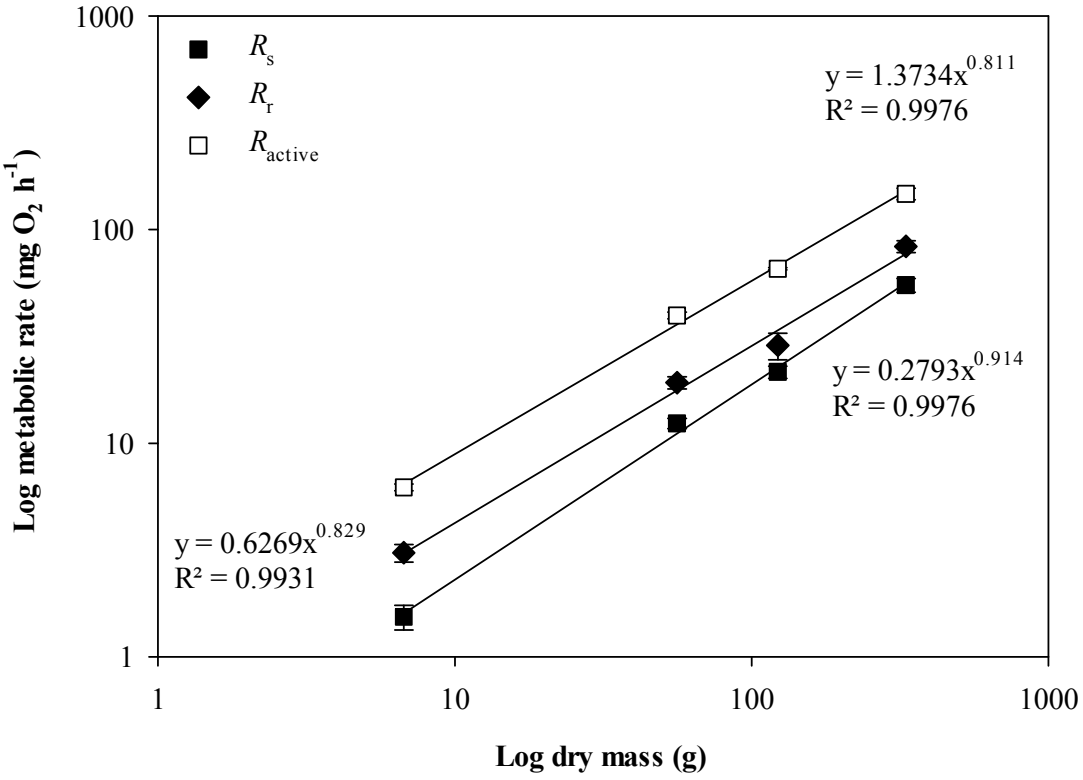

Figure 7.2. Allometric relationship between log of dry mass (DM) (g) and standard (R_s), routine (R_r), and active (R_{active}) metabolic rates (mg O_2 h^{-1}) of different sized *Sagmariasus verreauxi* juveniles. Values are mean (±SE).

7.4.1 Difference in the mass scaling exponent between species

In the current thesis, the overall regression for development of *S. verreauxi* resulted in a mass scaling exponent of 0.93 (Fig. 7.3), which is at the higher end of the range reported for fish, amphibians, and reptiles (Brett and Groves, 1979; Goolish, 1995; Clarke and Johnston, 1999; White et al., 2006, Fig. 7.4). However, the finding that the scaling exponent changes during ontogeny is an important consideration for comparative studies. The majority of previous studies that estimated species-specific scaling exponents did not cover as wide a mass range as in the current research. Therefore, previous studies may have calculated substantially different scaling exponents because they were not derived from the entire ontogenetic mass range (Moran and Wells, 2007). For instance, if the scaling exponent for *S. verreauxi* was determined during larval development the exponent would be 0.97, which is considerably higher than the 0.83 derived for juvenile development. This illustrates that it may also be more appropriate to examine scaling exponents between major life history stages separately for direct species comparisons (Moran and Wells, 2007), especially since there are so many morphological and physiological differences that occur between life stages of aquatic organisms (Oikawa et al., 1991). If a species comparison was made using the value of the juvenile *S. verreauxi* exponent this would be close to the exponent value reported across the mass range for larval to adult fish by White et al. (2006).

The allometric relationship obtained for juvenile *S. verreauxi* also conforms to the relationship found at optimum temperatures (0.8-0.9) in *Jasus lalandii* (Zoutendyk, 1989), but contrast those obtained in *Panulirus argus* (0.75) (Perera et al., 2007) and *J. edwardsii* (0.60) (Crear and Forteath, 2000). However, Perera et al. (2007) only examined a narrow mass range (50-270 g) and noted that temperature can

have a significant impact on the scaling exponent. Differences in scaling exponents may therefore be attributed to the thermal difference between tropical (*P. argus*), temperate (*J. edwardsii*), and warm temperate (*S. verreauxi*) lobster species and reflect metabolic differences between species. It may also be that species-specific differences are some of the largest factors affecting the allometric relationship of animals (Crear and Forteath, 2001).

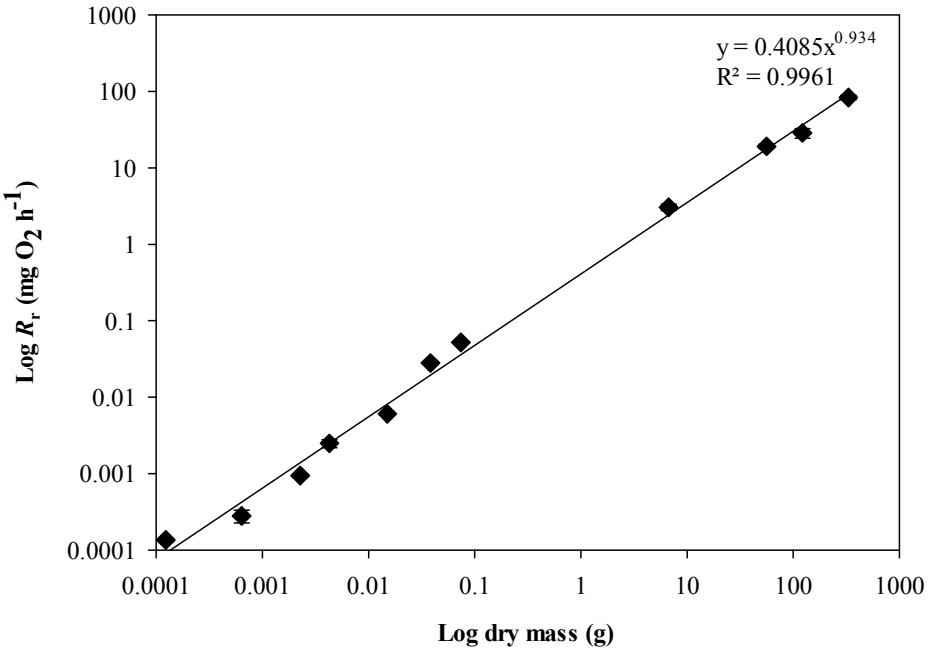

Figure 7.3. Allometric relationship between log of dry mass (DM) (g) and routine metabolic rate (R_r) (mg O_2 h^{-1}) of *Sagmariasus verreauxi* phyllosoma and juveniles during ontogeny. Values are mean (±SE).

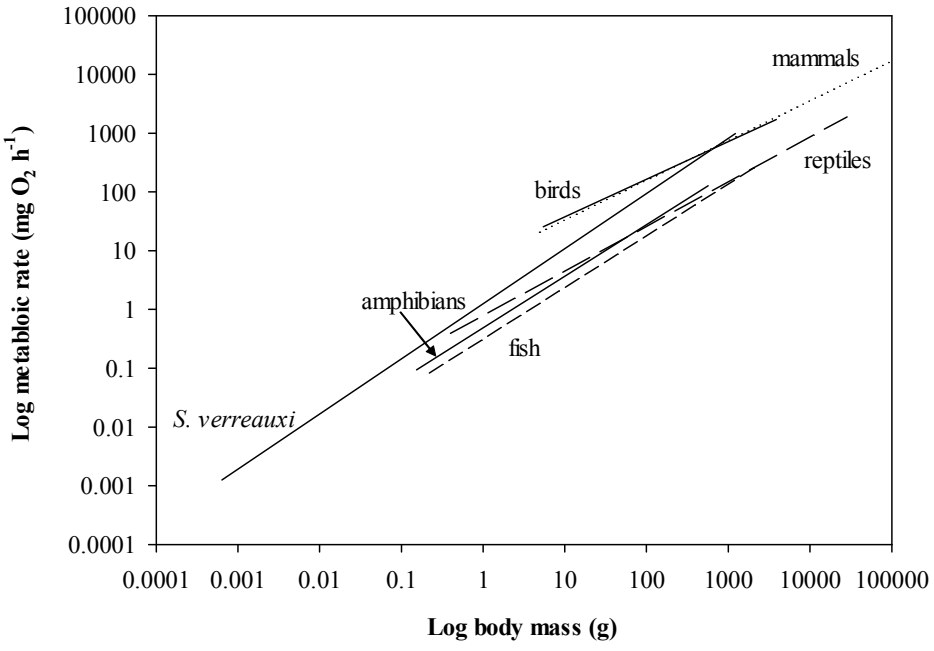

Figure 7.4. Intraspecific relationship between standard metabolic rate (R_s) (mg O_2 h^{-1}) normalised to a body temperature of 38°C and body mass (g) of fish, amphibians, reptiles, birds, and mammals from White et al. (2006). With the relationship between routine metabolic rate (R_r) (mg O_2 h^{-1}) and body mass (g) of *Sagmariasus verreauxi* included for comparison. Metabolic rates were normalised to a study temperature of 38°C with Q_{10} values of 1.65 (fish), 2.21 (amphibians), 2.44 (reptiles), 2.09 (birds), 2.81 (mammals), and 2.00 (*Sagmariasus verreauxi*).

7.5 Factorial aerobic scope

The ratio of R_{active} to R_s defines an animal's factorial aerobic scope (FAS) (Killen et al., 2007). This measure relates the factor by which individuals can increase their metabolic activity above maintenance levels, and hence, represents the capacity to support oxygen-consuming physiological functions and respond to challenges (Jobling, 1983; Priede, 1985; Bishop, 1999). The FAS of *S. verreauxi* (2.7-4.4; Fig. 7.5C) is similar to that previously recorded in other decapod crustaceans (McMahon et al., 1979; Booth et al., 1982; Crear and Forteath, 2000). However, this is considerably lower than the highest values (8-12 times R_s) recorded for various terrestrial crabs (Full and Herreid, 1983; Full, 1987; Maitland, 1987). The differences in FAS between *S. verreauxi* and various terrestrial crabs may be attributed to the more active nature of these crab species in their natural environment compared to relatively inactive benthic spiny lobsters (Crear and Forteath, 2000).

The effect of mass on R_s and R_{active} of *S. verreauxi* resulted in an overall decrease in FAS throughout juvenile development. There is very little information and comparative data, especially in lobsters, on the processes limiting FAS in larger individuals. However, these processes may be similar to those causing thermal sensitivity earlier in larger individuals, which is characterised by oxygen-limitation and reduced aerobic scope (Pörtner and Peck, 2010; Storch et al., 2011). Larger lobsters may be more limited by scaphognathite pumping frequency, branchial water flow and possibly even gill area causing less efficient gas exchange (McMahon et al., 1979) and subsequent lower body fluid oxygen levels compared to smaller lobsters (Pörtner, 2002). However, this cannot be confirmed because these parameters were not measured in my research. Smaller body size also enables higher oxygen uptake due to increased body surface area to volume ratios (Pörtner, 2002) and differences in

the thickness of the exoskeleton may limit respiratory gas exchange in larger lobsters (Spicer and Eriksson, 2003).

To my knowledge, changes in aerobic scope with body mass have not previously been measured in juvenile crustaceans. However, the processes limiting the aerobic scope of larger lobsters in comparison to smaller lobsters are most likely similar to those described for FAS. The range of aerobic scope for activity in *S. verreauxi* (0.28 – 0.69 mg O_2 g DM^{-1} h^{-1}; Fig. 7.5B) is higher than that measured for other large decapod crustaceans (Spoek, 1974; McMahon et al., 1979; Booth et al., 1982; Crear and Forteath, 2001), which may be a reflection of the different respirometry methods implemented in earlier studies. The respiratory methods used in some earlier studies may have acquired metabolic rates closer to R_r rather than R_s and the handling technique used to induce activity in the latter study may have underestimated R_{active}. Handling only causes a minor increase in $\dot{M}O_2$ of juvenile lobsters (see Chapter 5), whereas intensive chase protocols similar to the one employed for my research have been used effectively to exercise crustaceans to exhaustion (Booth and McMahon, 1992; Jimenez et al., 2008).

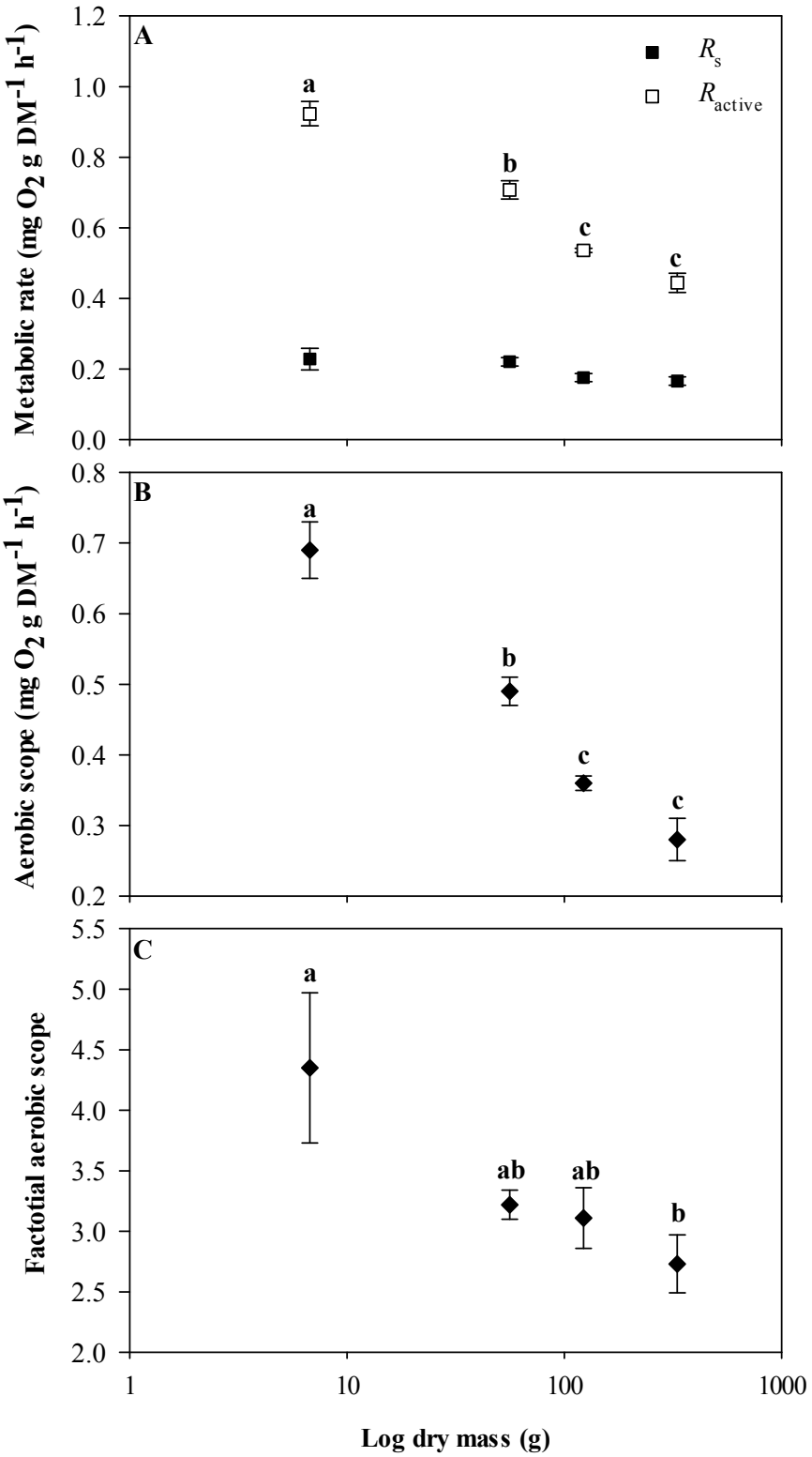

Figure 7.5. Changes in: (A) standard (R_s) and active (R_{active}) metabolic rates; (B) aerobic scope and; (C) factorial aerobic scope (FAS) of different sized *Sagmariasus verreauxi* juveniles plotted against log of dry mass (DM) (g). Data points bearing different superscripts are significantly different between mass classes (P<0.05). Values are mean (±SE).

7.6 Conclusions

The results of my research clearly demonstrate that the intraspecific scaling of R_r during ontogeny, and also R_s and R_{active} of juvenile *S. verreauxi*, differ considerably from the 0.75 (3/4) and 0.67 (1/3) mass dependent scaling exponents that are attributed to many animals. This indicates that metabolic rate is more dependent on body mass in lobsters and species-specific differences may be one of the largest factors affecting the allometric relationship of animals. Elevated scaling exponents were observed for larval development, which may be attributed to increased growth rates, which increase metabolic rates due to the high energetic cost of growth; the variable appearance of metabolically active tissues and organs as rapid growth of larval animals is accompanied by rapid development of organs with high metabolic activity; or increased respiratory surface area as they shift from diffusive respiration to the use of gills in later larval stages and juvenile development. Activity (R_{active}) also caused a reduction in the scaling exponent of juvenile lobsters compared to R_s. The FAS and aerobic scope decreased as a function of body mass in juvenile lobsters possibly because of differences in scaphognathite pumping frequency, branchial water flow and gill area causing less efficient gas exchange in larger lobsters.

There was a clear shift in energy substrate utilisation between phyllosoma and juvenile stages, which may reflect lifecycle changes in development from energy storage to tissue growth. Routine metabolic rate generally decreased during development, apart from an increase in later larval stages which was possibly due to increased activity and substantial physiological changes and high energy requirements of phyllosoma in preparation for metamorphosis. In summary, the long larval phase of *S. verreauxi* may be an evolutionary trait essential for accumulating lipid reserves to fuel later larval stages and metamorphosis. All evidence in this thesis indicates that

significant shifts in metabolic and biochemical physiology occur in later larval stages prior to metamorphosis. Successful culture of spiny lobsters should therefore aim to reduce energetic demands placed on phyllosoma during this highly critical phase and provide optimum environmental and culture conditions for accumulating the necessary energy reserves for metamorphosis and the puerulus stage. Future research should continue to focus on these factors in order to improve survival through metamorphosis and the puerulus stage and facilitate the continued increase in lobster propagation to meet the increasing demand for this seafood.

7.7 References

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